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MINIREVIEW

Non-antibiotics, Efflux Pumps and Drug Resistance of Gram-negative Rods

AGNIESZKA EWA LAUDY*

Department of Pharmaceutical Microbiology, Medical University of Warsaw, Warsaw, Poland

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Abstract

Non-antibiotic medicinal products consist of drugs with diverse activity against bacteria. Many non-antibiotics demonstrate direct antibacterial activity against Gram-positive cocci. The activity observed against Gram-negative rods is much lower and non-antibiotics primarily from the following groups: non-steroidal anti-inflammatory drugs, cardiovascular and antidepressant medicinal products demonstrate this activity. It has been shown that the low activity of some non-antibiotics or the absence of activity against Gram-negative rods is related, among other things, to the extrusion of these compounds from bacterial cells by multi-drug resistance efflux pumps. Substrates for the resistance-nodulation-division efflux systems include the following non-antibiotics: salicylate, diclofenac, ibuprofen, mefenamic acid, naproxen, amitriptyline, alendronate sodium, nicergoline, and ticlopidine. In addition, interactions between non-antibiotics and multi-drug resistance efflux pumps have been observed. It has also been revealed that depending on the concentration, salicylate induces expression of multi-drug resistance efflux pumps in *Escherichia coli*, *Salmonella enterica* subsp. *enterica* serotype Typhimurium, and *Burkholderia cenocepacia*. However, salicylate does not affect the expression of the resistance-nodulation-division efflux systems in *Stenotrophomonas maltophilia* and *Acinetobacter baumannii*. Most importantly, there were no effects of medicinal products containing some non-antibiotic active substances, except salicylate, as substrates of multi-drug resistance efflux pumps, on the induction of Gram-negative rod resistance to quinolones.

Key words: MDR efflux pumps, *Escherichia coli*, *Pseudomonas aeruginosa*, antibiotic resistance, non-antibiotics

Introduction

The resistance of Gram-negative rods to antibacterial compounds is related to the occurrence and interaction of several independent mechanisms of resistance. The following resistance mechanisms have been described in these rods: the production of various enzymes that inactivate antibiotics (*e.g.* β-lactams, aminoglycosides), active extrusion of bacterial compounds by membrane pumps (that govern resistance to fluoroquinolones, but the contribution of efflux pumps to β-lactams and tetracycline resistance has also been described), changes in the target sites of chemotherapeutic agents (*e.g.* fluoroquinolones, tetracyclines), alterations in outer membrane permeability that perturb the influx of antibiotics (*e.g.* some β-lactams), or involvement of additional metabolic pathways (primarily related to resistance to cotrimoxazole). Currently, the greatest therapeutic challenge is treatment of infections caused by Gram-negative rods producing β-lactam hydrolysing enzymes, *i.e.* metallo-β-lactamases (MBL), carbapenemases KPC-type (*Klebsiella pneumoniae* carbapenemases) and extended-spectrum β-lactamases (ESBL) (Miriagou *et al.*, 2010; Poirel *et al.*, 2012). However, the underestimated resistance mechanism of Gram-negative rods is an overexpression of multi-drug resistance (MDR) efflux pumps. In these rods the efflux pumps from all known five families are present, as follows: ABC (ATP-binding cassette family), RND (resistance-nodulation-division family), MFS (major facilitator superfamily), SMR (small multidrug resistance family), and MATE (multidrug and toxic compound extrusion family) (Piddock, 2006; Nikaido and Pages, 2012). The main role in resistance of rods plays the RND efflux systems that can simultaneously remove several different classes of antibiotics, biocides as well as organic compounds from bacterial cells. The RND proteins are encoded by genes organized in operons that are located in bacterial chromosomes (Piddock, 2006). Two new efflux pumps – OqxAB and QepA, encoded by genes located in conjugational plasmids, have recently been reported (Yamane *et al.*, 2008; Kim *et al.*, 2009). Expression of genes encoding efflux pumps as well as the efflux systems operons are regulated by both local and global regulator genes (Grkovic *et al.*, 2002; Piddock, 2006). In Gram-negative rods from the *Enterobacteriaceae* family, four groups of proteins (*e.g.* Mar, Sox, Rob and Ram) play role as global regulators of pump-encoding genes. The most important

* Corresponding author: A.E. Laudy, Department of Pharmaceutical Microbiology, Medical University of Warsaw, Warsaw, Poland; **e-mail: alaudy@wp.pl**

global regulator in *Pseudomonas aeruginosa* (non-fermentative Gram-negative rods) is the SoxR protein.

In bacteria, the presence of various resistance mechanisms, their interactions, and their complicity in conditioning the resistance of strains to antimicrobial compounds has resulted in increased difficulty in the treatment of infections as well as less effective treatment. The World Health Organization (WHO) in February 2017 published a list of the most dangerous bacterial pathogens, divided into 12 groups, which should be the priority of current research and new therapeutic options (WHO, 2017). The first group of these "critical" bacteria contains Gram-negative rods: *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacteriaceae*, which exhibit resistance to carbapenems. In addition, this group also includes *Enterobacteriaceae* strains, which produce ESBL enzymes. The WHO predicts that in the near future there may be a rapid increase in the number of infections caused by these rods, for which we no longer have effective therapeutic options. Therefore, the urgent challenge is to identify new groups of compounds with potential broad spectrum antimicrobial activity, especially against Gram-negative rods, which have recently been shown to be responsible for many life-threatening infections. For many years, research has been conducted to devise new therapeutic approaches for the treatment of bacterial infections, such as the manipulation of the host microbiome and the use of bacteriophages to kill bacteria.

An alternative to the search for new therapeutic options is the examination of so-called "non-antibiotics, which include medicines from various therapeutic groups used to treat diseases not related to microbial infections. The active substances of these drugs may also possess antibacterial activity (Martins *et al.*, 2008). However, most of the tested non-antibiotics have been shown to exhibit direct activity only against Gram-positive cocci. Considering the wide substrate range of RND efflux systems in Gram-negative bacteria (Laudy, 2008; Nikaido and Pages, 2012; Li *et al.*, 2015), the contribution of these pumps to the rods resistance to non-antibiotics was investigated (Laudy *et al.*, 2016; 2017). It is known that the reduction of susceptibility of Gram-negative rods to many antibiotics and disinfectants is due to the fact that they are substrates for MDR efflux pumps (Laudy, 2008; Nikaido and Pages, 2012; Li *et al.*, 2015).

Direct antibacterial activity of non-antibiotics

The group of non-antibiotic drugs may be divided into two subgroups, which differ in biological activity. The first subgroup consists of so-called antimicrobial non-antibiotics, which possess direct antibacterial activity. The second subgroup consists of two subclasses: the "helper compounds", which alter the permeability of bacteria to conventional antibiotics, and the "macrophage modulators", which enhance the cytotoxic activity of macrophages involved in bacterial phagocytosis (Martins *et al.*, 2008).

Most of the data published thus far relates to the direct antibacterial activity of non-antibiotics. However, it should be noted that the minimal inhibitory concentration (MIC) values of non-antibiotics against bacteria were not always determined in accordance with Clinical Laboratory Standard Institute (CLSI) recommendations and therefore some of these results may not be fully comparable. In addition, the studies were often conducted with only active substances of non-antibiotics and less frequently with the relevant medicinal products. The compounds tested belonged to various therapeutic groups, including anti-inflammatory drugs, cardiovascular drugs, antianaplastics, antiarrhythmics, anticonvulsants, antidepressants, antihypertensives, and spasmolytics. However, most of these non-antibiotic agents showed only marginal direct antibacterial activity (MIC ≥ 3000 mg/l) (Kruszewska *et al.*, 2008; 2010; Laudy *et al.*, 2016; 2017). Some of these compounds *e.g.* most phenothiazines (Kristiansen *et al.*, 2007), some antihistamines (Kruszewska *et al.*, 2002), anaesthetics (Kruszewska *et al.*, 2002), dodecyl(C(12)) gallate(3,4,5-trihydroxybenzoate) (Kubo *et al.*, 2003), and trans-chlorprothixene (Kristiansen *et al.*, 2010) were active only against Gram-positive cocci. However, for a few non-antibiotics a significant activity (MIC ≤ 800 mg/l) against both Gram-positive and Gram-negative bacteria has been described. These compounds include some phenothiazines (promazine (Hendricks *et al.*, 2003) and chloropromazine (Kristiansen *et al.*, 2010)), some cardiovascular drugs (Mazudar *et al.*, 2010), 2-dimethyl-amino-ethylchloride (Hendricks *et al.*, 2003), oxymetazoline (Kruszewska *et al.*, 2002), and sertraline (Kruszewska *et al.*, 2004).

The activity against Gram-negative rods of non-antibiotic active substances from the following groups: local anesthetics (*e.g.* lidocaine, bupivacaine, and ropivacaine) against *Escherichia coli* and *P. aeruginosa* (Tamanai-Shacoori *et al.*, 2007); locally vasoconstrictive agents (*e.g.* oxymetazoline) against *E. coli* (Kruszewska *et al.*, 2002), and proton pump inhibitors (*e.g.* rabeprazole and lansoprazole) against *Helicobacter pylori* (Bown, 2002) has also been reported. However, most compounds of the non-antibiotics group show only low activity against Gram-negative rods, *i.e.*, MIC values > 3000 mg/l (Kruszewska *et al.*, 2010).

Cardiovascular drugs are the group of non-antibiotics, to which special attention should be paid. Some of these drugs display high activity not only against Gram-positive cocci (*e.g. Staphylococcus aureus*), but

also against *Enterobacteriaceae* and non-fermentative Gram-negative rods (Mazumdar *et al.*, 2010). The highest activity (MICs of 10–200 mg/l) was demonstrated for the cardiovascular agents amlodipine, dobutamine, lacidipine, nifedipine, and oxyfedrine against the following Gram-negative bacteria: *E. coli* (2–25 strains were used in these studies), *Klebsiella* sp. (3–8 strains), *Salmonella* sp. (5–14 strains), *Shigella* sp. (12–42 strains), and *Pseudomonas* sp. (1–8 strains). In addition, the effects of these five cardiovascular agents in combination with various antibiotics against Gram-negative rods were analysed using *in vitro* tests, including the disc diffusion method, the checkerboard assay, and evaluation of the fractional inhibitory concentration (FIC) index. The synergism between tetracycline and oxyfedrine (FIC index 0.15) (Mazumdar *et al.*, 2005), and streptomycin and amlodipine (FIC index 0.28) (Asok *et al.*, 2004), was demonstrated against *Shigella dysenteriae* 7 NCTC 519/66. In another study, lacidipine showed synergism only with triflupromazine against *Salmomella enterica* subsp. *enterica* serotype Typhimurium NCTC 74 (Dasgupta *et al.*, 2010). However, these studies were conducted only on active substances of cardiovascular drugs.

The important observation has been published recently (Laudy *et al.*, 2017). The antidepressant agent amitriptyline and the relevant medicinal product (Amitriptylinum tabl.) have been shown to be active, with MIC values ranging from 100 to 800 mg/l, against all 180 studied clinical strains from species such as *K. pneumoniae*, *E. coli*, *P. aeruginosa*, *A. baumannii*, and *Stenotrophomonas maltophilia*. Moreover, in this study all clinical strains of *P. aeruginosa* and *S. maltophilia* were also susceptible (MICs ≤ 800 mg/l) to alendronate sodium, a specific inhibitor of osteoclast-mediated bone resorption, and the relevant medicinal product (Ostenil tabl.). It is worth emphasising that the MIC values of alendronate were ≤ 200 mg/l for 33/36 *P. aeruginosa* and 10/36 *S. maltophilia* strains studied.

More interesting non-antibiotics with potential antibacterial activity are the non-steroidal anti-inflammatory drugs (NSAIDs), which are among the most commonly and most widely used drugs in the world. The NSAID group includes compounds with different chemical structures; however, all of them show, in varying degrees, three biological activities: anti-inflammatory, analgesic, and antipyretic. The best-known substance in this group is diclofenac. The activity of the active substance diclofenac against the broad spectrum of Gram-negative rods, including *E. coli*, *Klebsiella* sp., *Salmonella* sp., *Shigella* sp., and *Vibrio cholerae*, has been described (Mazumdar *et al.*, 2006; Dutta *et al.*, 2007). In addition, the activity of diclofenac, both as an active substance alone and as a medicinal product containing diclofenac (Olfen tabl. and Diclac ini.), against all tested clinical strains of *K. pneumoniae*, *E. coli*, *Proteus mirabilis*, *P. aeruginosa*, *A. baumannii*, and *S. maltophilia*, with MIC values ranging from 800 to 3200 mg/l, has been demonstrated (Laudy *et al.*, 2016). Furthermore, it has been shown that diclofenac inhibits bacterial DNA synthesis (Dastidar *et al.*, 2000). Recently, the mechanism of action of the other small molecules of the NSAID group, including bromfenac, carprofen, and vedaprofen, has been demonstrated (Yin *et al.*, 2014). These compounds inhibited the *E. coli* DNA polymerase III b subunit, which disturbed DNA replication. Targeting the bacterial DNA replication machinery is a validated strategy for production of antibacterial chemotherapeutics like quinolones. In contrast to the fluoroquinolones, the NSAIDs that inhibit DNA replication exhibit weak antibacterial activity (Yin *et al.*, 2014).

Among the NSAIDs, the activity of acetylsalicylic acid against *E. coli* (Al-Bakri *et al.*, 2009; Laudy *et al.*, 2016), *P. aeruginosa*, *S. maltophilia*, *A. baumannii* (Laudy *et al.*, 2016), and *H. pylori* (Wang *et al.*, 2003) was also demonstrated. Furthermore, *H. pylori* demonstrated the increased sensitivity to antibiotics in the presence of acetylsalicylic acid (Wang *et al.*, 2003). The activity of ibuprofen and indometacin against *H. pylori* has also been demonstrated (Shirin *et al.*, 2006). Moreover, the activities of ibuprofen/Nurofen tabl. and naproxen/Naproxen tabl., as well as the active substances and medical products containing these agents against clinical strains of *S. maltophilia* (MICs 800–3200 mg/l) have been described (Laudy *et al.*, 2016).

Non-antibiotics as substrates of MDR efflux pumps

The cellular envelopes of Gram-negative rods contain MDR efflux pumps, which actively extrude harmful substances, such as antibiotics, chemotherapeutics, and disinfectants from bacteria. In contrast to pumps present in Gram-positive bacteria, MDR efflux pumps of Gram-negative rods extrude compounds of similar structures as well as several groups of substances that differ significantly from one another. The main role in the resistance of rods to antibiotics is played by the RND efflux systems, which exhibit wide substrate specificity (Laudy, 2008; Nikaido *et al.*, 2012; Li *et al.*, 2015). These systems, unlike the other MDR efflux pumps, have a wide substrate spectrum and can extrude many different antibacterial chemical compounds, such as antibiotics (mainly quinolones, tetracyclines, aminoglycosides, β-lactams, chloramphenicol, and erythromycin), disinfecting agents (*e.g.* triclosan), some aromatic hydrocarbons, acriflavine, rhodamine 6G, vanadium, crystal violet, and ethidium bromide. The best-known MDR efflux systems are MexAB-OprM (found in *P. aeruginosa*) and AcrAB-TolC (originally

described in *E. coli*, but also found in other species of the *Enterobacteriaceae* family) (Laudy, 2008; Nikaido *et al.*, 2012; Li *et al.*, 2015). Moreover, it has been shown that an overexpression of pump systems from the RND family causes resistance or reduced sensitivity of clinical strains, *e.g. P. aeruginosa* to fluoroquinolones (Kriengkauykiat *et al.*, 2005; Adabi *et al.*, 2015).

Considering the wide and diverse substrates range of the RND efflux systems, a study was conducted on the influence of MDR efflux pumps on the activity of non-antibiotics, both active substances and the corresponding drugs, against Gram-negative bacteria (Laudy *et al.*, 2016; 2017). Such a phenomenon was fully documented for salicylate, an essential NSAID belonging to non-antibiotics, which is a substrate of the CeoAB-OpcM efflux system in *Burkholderia cenocepacia* (Nair *et al.*, 2004). The increased accumulation of radiolabeled salicylate after the addition of 0.25 mM of the proton conductor, carbonyl cyanide m-chlorophenylhydrazone (CCCP), was observed. In the presence of CCCP, the bacterial cell membrane was de-energised. Furthermore, it was also observed that other NSAIDs, both active substances and relevant medicinal products, such as mefenamic acid/Mefacit tabl., ibuprofen/Nurofen tabl., naproxen/Naproxen tabl., diclofenac/Olfen tabl., and Diclac ini., were actively removed, most likely by MDR efflux pumps present in *Enterobacteriaceae* and in non-fermentative Gram-negative rods (Laudy *et al.*, 2016). This research was carried out by phenotypic methods using Phe-Arg-β-naphthylamide (PAβN), an inhibitor of efflux pumps that belongs to the RND family (Lomovskaya *et al.*, 2001). It is known that PAβN potently inhibits efflux systems of the Mex family in *P. aeruginosa* (especially MexAB-OprM) and inhibits the AcrAB-TolC efflux system of the *Enterobacteriaceae* family (*e.g. E. coli*, *K. pneumoniae*, *P. mirabilis*, *Enterobacter aerogenes*, and *S. enterica* subsp. *enterica* serotype Typhimurium) (Lomovskaya *et al.*, 2001; Pagès and Amaral, 2009; Nikaido *et al.*, 2012; Li *et al.*, 2015). An *in vitro* phenotypic screening of bacteria for antibiotic removal by MDR efflux pumps is based on measurement of changes in the MICs values of antibiotic in the absence or presence of the efflux pump inhibitor (Lomovskaya *et al.*, 2001; Kriengkauykiat *et al.*, 2005; Adabi *et al.*, 2015; Laudy *et al.*, 2015). Significant decreases (≥ 4-fold) in the MIC values of the NSAID non-antibiotics: mefenamic acid/Mefacit tabl., ibuprofen/Nurofen tabl., naproxen/Naproxen tabl., diclofenac/Olfen tabl., and Diclac ini. in the presence of PAβN was demonstrated among majority of clinical strains of *K. pneumoniae*, *E. coli*, *P. mirabilis*, *P. aeruginosa*, *A. baumannii*, and *S. maltophilia* (Laudy *et al.*, 2016). In the presence of PAβN, the highest increase in bacterial susceptibility to NSAIDs was observed for diclofenac and mefenamic acid and the relevant medicinal products when the isolates of *S. maltophilia* (MICs of 25–1000 and 100 mg/l, respectively) and *E. coli* (MICs of 50 and 100 mg/l, respectively) were studied. In addition, significant increases in the susceptibility of *E. coli* and *P. mirabilis* clinical strains to acetylsalicylic acid/Aspirin tabl. were shown in the presence of PAβN (Laudy *et al.*, 2016).

The research was also conducted with non-antibiotics from other non-NSAIDs therapeutic groups. The impact of PAβN on the susceptibility of bacteria to active substances and the relevant medicinal products, such as amitriptyline/Amitriptylinum tabl., alendronate sodium/Ostenil tabl., nicergoline/Niglostin tabl., and ticlopidine/Apo-Clodin tabl. was observed and it suggested that these non-antibiotics were substrates of efflux pumps in *Enterobacteriaceae* and non-fermentative Gram-negative rods (Laudy *et al.*, 2017). For amitriptyline/Amitriptylinum tabl. and alendronate sodium/Ostenil tabl., significant decreases (≥ 4-fold) in the MIC values of these non-antibiotics in the presence of PAβN were demonstrated for most of *K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *A. baumannii* (only for amitriptyline/Amitriptylinum tabl.) clinical strains. Similarly, this phenomenon has been observed for *K. pneumoniae*, *E. coli*, *A. baumannii*, and *S. maltophilia* strains when ticlopidine/Apo-Clodin tabl were used. However, significant decreases in the MIC values of nicergoline/Niglostin tabl. were only shown for strains of *A. baumannii*.

An interesting observation was made for ticlopidine. This agent inhibits cellular teichoic acid synthesis and blocks the activity of penicillin-binding proteins, which results in the susceptibility of methicillin-resistant *S. aureus* strains to β-lactams (Farha *et al.*, 2013). Ticlopidine does not possess antibacterial activity but displays potential synergistic activity with cefuroxime against Gram-positive cocci (Farha *et al.*, 2013). A particularly important observation was the restoration of susceptibility to ticlopidine/Apo-clodin tabl. in the presence of PAβN among clinical strains of *E. coli*, *K. pneumoniae*, *A. baumannii*, and *S. maltophilia* (Laudy *et al.*, 2017), indicating a different mechanism of ticlopidine action against Gram-negative rods compared with Gram-positive bacteria.

The presence of MDR pumps could be an important factor that contributes to lack of or poor activity of some non-antibiotics against Gram-negative rods.

Interactions between non-antibiotics and MDR efflux pumps

An important and interesting issue is the influence of non-antibiotic medicinal products on efficient treatment of bacterial infections in the context

of drug interactions with bacterial MDR efflux pumps. As early as the 1980s, the influence of salicylates (*e.g.* sodium salicylate and acetylsalicylic acid) on the induction of resistance to chloramphenicol, nalidixic acid, tetracycline, ampicillin, and cephalosporins was demonstrated with reference *E. coli* strains K-12 and JF568 (Rosner, 1985; Foulds *et al.*, 1989). Since then, the salicylate-associated increase in antibiotic resistance has also been described for other Gram-negative rods, including *K. pneumoniae* (Domenio *et al.*, 1990), *S. enterica* subsp. *enterica* serotype Typhimurium (Hartog *et al.*, 2010), and *B. cenocepacia* (Nair *et al.*, 2004). Thus, the question arises whether different groups of non-antibiotics may affect the activity of bacterial RND efflux systems and thus modify the susceptibility of Gram-negative rods to antibiotics.

Among the non-antibiotics, salicylates have been thoroughly investigated, including their influence on the expression of genes encoding MDR efflux pumps and the change of bacterial sensitivity to antibiotics. Acetylsalicylic acid in humans is rapidly hydrolysed to salicylic acid in the stomach and liver (Needs and Brooks 1985).

The salicylate-induced antibiotic resistance in *E. coli* is due to the increase of transcription level of the *marRAB* operon, which encodes the MarA protein (Cohen *et al.*, 1993). This increased production of the global regulator MarA enhances the transcription of the *acrAB* operon. Consequently, this leads to overexpression of the multidrug AcrAB-TolC efflux system. Substrates for this efflux system include quinolones, tetracyclines, chloramphenicol, tigecycline, rifampicin, fusidic acid, oxazolidinones, macrolides, and some β-lactams (Laudy, 2008; Nikaido *et al.*, 2012; Li *et al.*, 2015). Salicylate can also affect the other two MDR efflux pumps of *E. coli*, EmrKY (Tanabe *et al.*, 1997; Price *et al.*, 2000) and EmrAB (Lomovskaya *et al.*, 1995; Price *et al.*, 2000). The ability to extrude tetracycline with the EmrKY efflux pump and nalidixic acid with the EmrAB-TolC efflux system has been demonstrated.

Importantly, induction of *marRAB* operon expression by salicylate is concentration-dependent (Cohen *et al.*, 1993). Salicylate at a concentration in the range of 0.01–0.1 mM, did not induce the *mar* promoter; however, at salicylate concentrations above 0.5 mM, the expression of *marRAB* was demonstrated (Cohen *et al.*, 1993). It was assumed that a therapeutic level of salicylate was up to 1.8 mM in the plasma (Wang *et al.*, 2003); and thus, at the concentration of 5 mM the observed salicylate-induced *E. coli* antibiotic resistance had limited therapeutic value (Rosner, 1985; Cohen *et al.*, 1993). The recommended levels of acetylsalicylic acid/Aspirin tabl. in plasma are in the range of 20–100 mg/l (0.1–0.55 mM) for analgesia and 150–300 mg/l (0.83–1.67 mM) for an anti-inflammatory effect. However, acetylsalicylic acid at a concentration of 2 mM is recommended to cure chronic inflammatory diseases, such as rheumatoid arthritis (Axon and Huskisson, 1992). It seemed that acetylsalicylic acid and salicylate only at concentrations of 5 mM or higher were toxic to humans (Frantz and O'Neill, 1995; Wu, 2000), but it has recently been shown that salicylate plasma levels higher than 2.2 mM are potentially toxic for patients chronically treated with salicylate (Wang *et al.*, 2003).

In contrast to the results obtained for *S. enterica* subsp. *enterica* serotype Typhimurium and *E. coli*, salicylate showed no significant impact on the expression of the operons *adeFGH* and *adeIJK* (encoding AdeFGH and AdeIJK efflux pumps, respectively, both from the RND family) in *A. baumannii* (Bazyleu and Kumar, 2014) and the *emrRCABsm* operon, encoding the EmrCABsm efflux pump belonging to the MFS family in *S. maltophilia* (Huang *et al.*, 2013). Moreover, expression of the *adeABC* operon, which encodes the AdeABC RND efflux pump in *A. baumannii,* at a high concentrations of salicylate (2.5–4 mM), was reduced 2.5-fold and did not show in the case of this strain the influence on the susceptibility level of ciprofloxacin, gentamicin, and ceftriaxone (Bazyleu and Kumar, 2014).

Recently, the influence of medicinal products containing non-antibiotics other than salicylates (which are likely extruded by efflux pumps), with or without PAβN, on the susceptibility of different species of Gram-negative rods to quinolones was investigated. Quinolones in these studies served as an example of compounds actively removed by efflux pumps. The participation of efflux pumps in resistance to fluoroquinolones has been shown in a variety of Gram-negative rod genera (Laudy, 2008; Nikaido and Pages, 2012; Adabi *et al.*, 2015). There were no effects of medicinal products containing the following active substances: alendroniane sodium, carboplatin, ticlopidine, nicergoline, amitriptyline, and NSAIDs, such as diclofenac, mefenamic acid, ibuprofen, and naproxen, for the induction of quinolone resistance of Gram-negative rods (*P. aeruginosa*, *S. maltophilia*, *A. baumannii*, *E. coli*, *K. pneumoniae*, and *P. mirabilis*) (Laudy *et al.*, 2016; 2017). The non-antibiotics, with the exception of salicylates, are substrates of MDR efflux pumps; however, they do not affect the sensitivity of Gram-negative rods and can be used safely in the treatment of bacterial infections.

Summary

Knowledge regarding antimicrobial activity of non-antibiotics is still limited. The first publications from the 1980s and 1990s concerned only a few active substances, such as salicylates. Recently, direct antibacterial activity of a broad spectrum of non-antibiotics (both

active substances and the relevant medical products) has been demonstrated *in vitro.* In addition, the active substances of the non-antibiotic group, including NSAIDs, antidepressants, antiplatelet drugs, and specific inhibitors of osteoclast-mediated bone resorption were found to be substrates of Gram-negative rod efflux pumps. The use of a PAβN pump inhibitor increased the sensitivity of clinical strains to the aforementioned non-antibiotics. The presence of RND efflux systems causes lack or low activity of non-antibiotics against Gram-negative rods. Among the non-antibiotics, only salicylates can induce the expression of operons encoding pump systems and efflux-dependent resistance to antibiotics in *E. coli*, *S. enterica* subsp. *enterica* serotype Typhimurium and *B. cenocepacia*. The noticeable impact of MDR efflux pumps on the resistance of Gram-negative rods to non-antibiotics as well as to classic antibiotics emphasizes the urgent need to look for inhibitors of these pumps that could be used in therapy. Despite extensive scientific research, also conducted by pharmaceutical companies, an efflux pump inhibitor that is not toxic to humans and can be applied in antibacterial therapy has not yet been discovered.

Literature

Adabi M., M. Talebi-Taher, L. Arbabi, M. Afshar, S. Fathizadeh, S. Minaeian, N. Moghadam-Maragheh and A. Majidpour. 2015. Spread of efflux pump overexpressing-mediated fluoroquinolone resistance and multidrug resistance in *Pseudomonas aeruginosa* by using an efflux pump inhibitor. *Infect. Chemother.* 47(2): 98–104.

Al-Bakri A.G., G. Othman and Y. Bustanji. 2009. The assessment of the antibacterial and antifungal activities of aspirin, EDTA and aspirin-EDTA combination and their effectiveness as antibiofilm agents. *J. Appl. Microbiol.* 107(1): 280–286.

Asok Kumar K., K. Mazumdar, N.K. Dutta, P. Karak, S.G. Dastidar and R. Ray. 2004. Evaluation of synergism between the aminoglycoside antibiotic streptomycin and the cardiovascular agent amlodipine. *Biol. Pharm. Bull.* 27(7): 1116–1120.

Axon J.M.C. and E.C. Huskisson 1992. Use of aspirin in inflammatory diseases, pp. 295–320. In: Vane J.R. and R.M. Botting (eds). *Aspirin and Other Salicylates*. Chapman and Hall, London (United Kingdom).

Bazyleu A. and A. Kumar. 2014. Incubation temperature, osmolarity, and salicylate affect the expression of resistance-nodulation-division efflux pumps and outer membrane porins in *Acinetobacter baumannii* ATCC 19606T. *FEMS Microbiol. Lett.* 357(2): 136–143.

Bown R.L. 2002. An overview of the pharmacology, efficacy, safety and cost-effectiveness of lansoprazole. *Int. J. Clin. Pract.* 56(2): 132–139.

Cohen S.P., S.B. Levy, J. Foulds and J.L. Rosner. 1993. Salicylate induction of antibiotic resistance in *Escherichia coli*: activation of the mar operon and a mar-independent pathway. *J. Bacteriol.* 175(24): 7856–7862.

Dastidar S.G., K. Ganguly, K. Chaudhuri and A.N. Chakrabarty. 2000. The anti-bacterial action of diclofenac shown by inhibition of DNA synthesis. *Int. J. Antimicrob. Agents.* 14(3): 249–251.

Dasgupta A., S. Chaki, S. Mukherjee, J. Lourduraja, K. Mazumdar, N.K. Dutta and S.G. Dastidar. 2010. Experimental analyses of synergistic combinations of antibiotics with a recently recognised antibacterial agent, lacidipine. *Eur. J. Clin. Microbiol. Infect. Dis.* 29(2): 239–243.

Domenico P., T. Hopkins and B.A. Cunha. 1990. The effect of sodium salicylate on antibiotic susceptibility and synergy in *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* 26(3): 343–351.

Dutta N.K., S. Annadurai, K. Mazumdar, S.G. Dastidar, J.E. Kristiansen, J. Molnar, M. Martins and L. Amaral. 2007. Potential management of resistant microbial infections with a novel non-antibiotic: the anti-inflammatory drug diclofenac sodium. *Int. J. Antimicrob. Agents.* 30(3): 242–249.

Farha M.A., A. Leung, E.W. Sewell, M.A. D'Elia, S.E. Allison, L. Ejim, P.M. Pereira, M.G. Pinho, G.D. Wright and E.D. Brown. 2013. Inhibition of WTA synthesis blocks the cooperative action of PBPs and sensitizes MRSA to β-lactams. *ACS Chem. Biol.* 8(1): 226–233.

Foulds J., D.M. Murray, T. Chai and J.L. Rosner. 1989. Decreased permeation of cephalosporins through the outer membrane of *Escherichia coli* grown in salicylates. *Antimicrob. Agents Chemother.* 33(4): 412–417.

Frantz B. and E.A. O'Neill. 1995. The effect of sodium salicylate and aspirin on NF-kappa *B. Science* 270(5244): 2017–2019.

Grkovic S., M.H. Brown and R.A. Skurray 2002. Regulation of bacterial drug export systems. *Microbiol. Mol. Biol. Rev.* 66 (4): 671–701.

Hartog E., O.Menashe, E. Kler and S. Yaron. 2010. Salicylate reduces the antimicrobial activity of ciprofloxacin against extracellular *Salmonella enterica* serovar Typhimurium, but not against *Salmonella* in macrophages. *J. Antimicrob. Chemother.* 65(5): 888–896.

Hendricks O., T.S. Butterworth and J.E. Kristiansen. 2003. The *in vitro* antimicrobial effect of non-antibiotics and putative inhibitors of efflux pumps on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Int. J. Antimicrob. Agents.* 22(3): 262–264.

Huang Y.W., R.M. Hu, F.Y. Chu, H.R. Lin and T.C. Yang. 2013. Characterization of a major facilitator superfamily (MFS) tripartite efflux pump EmrCABsm from *Stenotrophomonas maltophilia*. *J. Antimicrob. Chemother.* 68(11): 2498–2505.

Kim H.B., M. Wang, C.H. Park, E.C. Kim, G.A. Jacoby and D.C. Hooper. 2009. oqxAB encoding a multidrug efflux pump in human clinical isolates of *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* 53(8): 3582–3584.

Kriengkauykiat J., E. Porter, O. Lomovskaya and A. Wong-Beringer. 2005. Use of an efflux pump inhibitor to determine the prevalence of efflux pump-mediated fluoroquinolone resistance and multidrug resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 49(2): 565–570.

Kristiansen J.E., O. Hendricks, T. Delvin, T.S. Butterworth, L. Aagaard, J.B. Christensen, V.C. Flores and H. Keyzer. 2007. Reversal of resistance in microorganisms by help of non-antibiotics. *J. Antimicrob. Chemother.* 59(6): 1271–1279.

Kristiansen J.E., V.F. Thomsen, A. Martines, M. Viveiros and L. Amaral. 2010. Non-antibiotics reverse resistance of bacteria to antibiotics. *In Vivo.* 24(5): 751–754.

Kruszewska H., T. Zaręba and S. Tyski. 2002. Search of antimicrobial activity of selected non-antibiotics drugs. *Acta Pol. Pharm. Drug Res.* 59(6): 436–439.

Kruszewska H., T. Zaręba and S. Tyski. 2004. Examination of antimicrobial activity of selected non-antibiotics drugs. *Acta Pol. Pharm. Drug Res.* 61(Suppl. 5): 18–21.

Kruszewska H., T. Zaręba and S. Tyski. 2008. Examination of antibacterial and antifungal activity of selected non-antibiotic products. *Acta Pol. Pharm. Drug Res.* 65(6): 779–782.

Kruszewska H., T. Zaręba and S. Tyski. 2010. Examination of antimicrobial activity of selected non-antibiotic products. *Acta Pol. Pharm. Drug Res.* 67(6): 733–736.

Kubo I., K. Fujita, K. Nihei and N. Masuoka. 2003. Non-antibiotic antibacterial activity of dodecyl gallate. *Bioorg. Med. Chem.* 11(4): 573–580.

Lomovskaya O., K. Lewis and A. Matin. 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. *J. Bacteriol.* 177(9): 2328–2334.

Lomovskaya O., M.S. Warren, A. Lee, J. Galazzo, R. Fronko, M. Lee, J. Blais, D. Cho, S. Ckamberland, T. Renau and others. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *Antimicrob. Agents Chemother.* 45(1): 105–116.

Laudy A.E. 2008. MDR efflux pumps – the mechanism of Gram-negative rods resistance to antibiotics. *Post. Mikrobiol.* 47(3): 415–422.

Laudy A.E., E. Kulińska and S. Tyski. 2017. The impact of efflux pump inhibitors on the activity of selected non-antibiotic medicinal products against Gram-negative bacteria. *Molecules.* 22(1): E114. doi:10.3390/molecules22010114.

Laudy A.E., A. Mrówka, J. Krajewska and S. Tyski. 2016. The influence of efflux pump inhibitors on the activity of non-antibiotic NSAIDS against Gram-Negative rods. *PLoS ONE.* 11(1): e0147131. doi:10.1371/journal.pone.0147131.

Laudy A.E., P. Osińska, A. Namysłowska, O. Zając and S. Tyski. 2015. Modification of the susceptibility of Gram-negative rods producing ESβLs to β-lactams by the efflux phenomenon. *PLoS ONE.* 10(3): e0119997. doi:10.1371/journal.pone.0119997.

Li X.Z., P. Plesiat and H. Nikaido. 2015. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin. Microbiol. Rev.* 28(2): 337–418.

Martins M., S.G. Dastidar, S. Fanning, J.E. Kristiansen, J. Molnar, J.M. Pages, Z. Schelz, G. Spengler, M. Viveros and L. Amaral. 2008. Potential role of non-antibiotics (helper compounds) in the treatment of multidrug-resistant Gram-negative infections: mechanisms for their direct and indirect activities. *Int. J. Antimicrob. Agents.* 31(3): 198–208.

Mazumdar K., K. Asok Kumar and N.K. Dutta. 2010. Potential role of the cardiovascular non-antibiotic (helper compound) amlodipine in the treatment of microbial infections: Scope and hope for the future. *Int. J. Antimicrob. Agents.* 36(4): 295–302.

Mazumdar K., N.K. Dutta, S.G. Dastidar, N. Motohashi and Y. Shirataki. 2006. Diclofenac in the management of *E. coli* urinary tract infections. *In Vivo* 20(5): 613–620.

Mazumdar K., N.K. Dutta, K.A. Kumar and S.G. Dastidar. 2005. *In vitro* and *in vivo* synergism between tetracycline and the cardiovascular agent oxyfedrine HCl against common bacterial strains. *Biol. Pharm. Bull.* 28(4): 713–717.

Miriagou V., G. Cornaglia, M. Edelstein, I. Galani, C.G. Giske, M. Gniadkowski, E. Malamou-Lada, L. Martinez-Martinez, F. Navarro, P. Nordmann and others. 2010. Acquired carbapenemases in Gram-negative bacterial pathogens: detection and surveillance issues. *Clin. Microbiol. Infect.* 16(2): 112–122.

Nair B.M., K.J. Cheung Jr., A. Griffith and J.L. Burns. 2004. Salicylate induces an antibiotic efflux pump in *Burkholderia cepacia* complex genomovar III (*B. cenocepacia*). *J. Clin. Invest.* 113(3): 464–473.

Needs C.J. and P.M. Brooks. 1985. Clinical pharmacokinetics of the salicylates. *Clin. Pharmacokinet.* 10(2): 164–177.

Nikaido H. and J.M. Pages. 2012. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol. Rev.* 36(2): 340–363.

Pagès J.M. and L. Amaral. 2009. Mechanisms of drug efflux and strategies to combat them: challenging the efflux pump of Gram-negative bacteria. *Biochim. Biophys. Acta.* 1794(5): 826–833.

Piddock L.J. 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin. Microbiol. Rev.* 19(2): 382–402.

Poirel L., R.A. Bonnin and P. Nordmann. 2012. Genetic support and diversity of acquired extended-spectrum β-lactamases in Gram-negative rods. *Infect. Genet. Evol.* 12(5): 883–893.

Price C.T., I.R. Lee and J.E. Gustafson. 2000. The effects of salicylate on bacteria. *Int. J. Biochem. Cell Biol.* 32(10): 1029–1043.

Shirin H., S.F. Moss, S. Kancherla, K. Kancherla, P.R. Holt, I.B. Weinstein and E.M. Sordillo. 2006. Non-steroidal anti-inflammatory drugs have bacteriostatic and bactericidal activity against *Helicobacter pylori*. *J. Gastroenterol. Hepatol.* 21(9): 1388–1393.

Rosner J.L. 1985. Nonheritable resistance to chloramphenicol and other antibiotics induced by salicylates and other chemotactic repellents in *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA* 82(24): 8771–8774.

Tanabe H., K. Yamasak, M. Furue, K. Yamamoto, A. Katoh, M. Yamamoto, S. Yoshioka, H. Tagami, H.A. Aiba and R. Utsumi. 1997. Growth phase-dependent transcription of emrKY, a homolog of multidrug efflux emrAB genes of *Escherichia coli*, is induced by tetracycline. *J. Gen. Appl. Microbiol.* 43(5): 257–263.

Tamanai-Shacoori Z., V. Shacoori, A. Jolivet-Gougeon, J.M. Vo Van, M. Repere, P. Donnio and M. Bonnaure-Mallet. 2007. The antibacterial activity of tramadol against bacteria associated with infectious complications after local or regional anesthesia. *Anesth. Analg.* 105(2): 524–527.

Wang W.H., W.M. Wong, D. Dailidiene, D.E. Berg, Q. Gu, K.C. Lai, S.K. Lam and B.C. Wong. 2003. Aspirin inhibits the growth of *Helicobacter pylori* and enhances its susceptibility to antimicrobial agents. *Gut.* 52(4): 490–495.

WHO (World Health Organization). 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf?ua=1, 2018.03.24.

Wu K.K. 2000. Aspirin and salicylate, an old remedy with a new twist. *Circulation.* 102(17): 2022–2023.

Yamane K., J. Wachino, S. Suzuki and Y. Arakawa. 2008. Plasmid-mediated *qepA* gene among *Escherichia coli* clinical isolates from Japan. *Antimicrob. Agents Chemother.* 52(4): 1564–1566.

Yin Z., Y. Wang, L.R. Whittell, S. Jergic, M. Liu, E. Harry, N.E. Dixon, M.J. Kelso, J.L. Beck and A.J. Oakley. 2014. DNA replication is the target for the antibacterial effects of nonsteroidal anti-inflammatory drugs. *Chem. Biol.* 21(4): 481–487.

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MINIREVIEW

Bacteriological, Clinical and Virulence Aspects of *Aeromonas*-associated Diseases in Humans

UTTARA DEY BHOWMICK and SOUMEN BHATTACHARJEE*

Cell and Molecular Biology Laboratory, Department of Zoology, University of North Bengal, Raja Rammohunpur, Siliguri, District Darjeeling, West Bengal, India

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Abstract

Aeromonads have been isolated from varied environmental sources such as polluted and drinking water, as well as from tissues and body fluids of cold and warm-blooded animals. A phenotypically and genotypically heterogenous bacteria, aeromonads can be successfully identified by ribotyping and/or by analysing gyrB gene sequence, apart from classical biochemical characterization. Aeromonads are known to cause scepticemia in aquatic organisms, gastroenteritis and extraintestinal diseases such as scepticemia, skin, eye, wound and respiratory tract infections in humans. Several virulence and antibiotic resistance genes have been identified and isolated from this group, which if present in their mobile genetic elements, may be horizontally transferred to other naive environmental bacteria posing threat to the society. The extensive and indiscriminate use of antibiotics has given rise to many resistant varieties of bacteria. Multidrug resistance genes, such as NDM1, have been identified in this group of bacteria which is of serious health concern. Therefore, it is important to understand how antibiotic resistance develops and spreads in order to undertake preventive measures. It is also necessary to search and map putative virulence genes of *Aeromonas* for fighting the diseases caused by them. This review encompasses current knowledge of bacteriological, environmental, clinical and virulence aspects of the *Aeromonas* group and related diseases in humans and other animals of human concern.

Key words: Aeromonad, diarrhea, multi-drug, resistance, virulence

Introduction

Aeromonads are recognized not only as an important disease-causing pathogen of fish and other cold-blooded organisms but also as a causative organism in a variety of infectious complications in both immunocompetent and immunocompromised humans. The name *Aeromonas* is derived from Greek noun *aeros* (air, gas) and *monas* (unit). Members of the genus *Aeromonas* can be referred to as aeromonad. Aeromonads (Phylum *Proteobacteria*, Class *Gammaproteobacteria*, Order *Aeromonadales*, Family *Aeromonadaceae*) are Gram-negative, non-spore forming, rod shaped, facultative anaerobic bacteria that occur in natural water bodies of the environment. They are similar in many characters to *Enterobacteriaceae* family.

The DNA-DNA hybridization studies showed the presence of 33 DNA hybridization groups, including 19 genospecies. *Aeromonas hydrophila*, *A. caviae*, *A. sobria*, *A. veronii*, and *A. schubertii* are mesophilic, whereas, *A. salmonicida* are non-motile and psychrophilic. Widely distributed, aeromonads have been isolated from various sources like freshwater fishes, drinking water supply, environmental samples, polluted waters, food items like meat, fish, milk, ready to eat items and oysters (Abeyta *et al*., 1986; Altwegg *et al*., 1990; Manna *et al*., 2013, Figueras *et al*., 2017). *Aeromonas* have been found in the *Aedes aegyptii* and *Culex quinque fasciatus* mosquitoes' midgut, in monkey faeces and bivalve molluscs (Pidiyar *et al.,* 2002), larvae of *Chironomus plumosus* (Rouf and Rigney, 1993).

Over the past few years, researchers have renewed interest in the genus *Aeromonas* as an emergent human pathogen (Janda and Abbott, 1998). Aeromonads have been implicated in septicaemia in variety of aquatic organisms and gastrointestinal/extra-intestinal diseases in humans (Janda and Duffey, 1988; Janda and Abbott, 1996). Several species of genus *Aeromonas* have been implicated in pathogenic cases in human, like cellulitis, surgical wound infections, nosocomial pneumonia, hemolytic-uremic syndrome, sepsis, peritonitis, meningitis, urinary tract infections, and severe muscle degeneration. In all the cases it seems that *Aeromonas*-mediated pathogenesis occurs both in cases of

* Corresponding author: S. Bhattacharjee, Cell and Molecular Biology Laboratory, Department of Zoology, University of North Bengal, Raja Rammohunpur, Siliguri, District Darjeeling, West Bengal, India; **e-mail: soumenb123@rediffmail.com, sbhnbu@gmail.com**

immunosuppression and immunocompetence (Wang *et al*., 2003). However, *Aeromonas*-mediated mechanism of pathogenesis in both aquatic organisms and in human subjects remains to be elucidated.

Aeromonas spp. possess multifactorial virulence genes and systems. Several groups have demonstrated the presence of aerolysin (Chakraborty *et al*., 1986), hemolysin (Wang *et al*., 1996), extracellular lipase (Anguita *et al*., 1993), cytolytic enterotoxin (Chopra *et al*., 1993), haemolytic toxin genes (Khan *et al*., 1998), acetylcholinesterase (Nieto *et al*., 1991) and proteases (Leung and Stevenson, 1988). Genome level scans have identified virulence factors in potential open reading frames (ORFs) and few putative genes, like O-antigen and capsule, gene cluster in phage and type III secretion system have been associated with virulent aeromonads. Several genomic islands (GIs) with unusual G-C content, have also been identified that carry mobility-associated genes, such as integrases or transposes and other putative virulence genes (Yu *et al*., 2005). *Aeromonas luxRI* quorum sensing gene homologs and Ribonuclease R (Vac B) have also been implicated in modulation and expression of these virulence genes (Jangid *et al*., 2007; Erova *et al*., 2008). The NDM-1 gene ($bla_{\text{NDM-1}}$) has been found in aeromonads of North India (New Delhi) (Walsh *et al*., 2011).

Aeromonads are found to inhabit a variety of niches including soil, aquatic habitats, aquatic animals, terrestrial animals, birds, insects, and human beings (Table I). *A. hydrophila* are found to inhabit a wide range of thermal and pH conditions, except in extremely polluted and saline water and hot water springs. Estuaries are ideal for *Aeromonas,* where they either exist freely or associated with crustaceans (Fiorentini *et al*., 1998). Most of the aeromonads come into human systems through ingestion of water or food contaminated with *Aeromonas*. In India, *Aeromonas* spp. have been detected in 13.4% of animal-origin food samples, the highest being in fish (Kumar *et al*., 2000). Aeomonads mostly infect the gastrointestinal tract, urinary tract and blood of human beings. Three *Aeromonas* species viz., *A. hydrophila*, *A. caviae* and *A. veronii* bv. Sobria are known to infect human beings (Janda and Abbott, 1998). Some other species like *A. jandaei*, *A. veronii* bv. *veronii*, *A. schubertii*, *A. popoffi* are also known to infect human (Janda *et al*., 1994; Hua *et al*., 2004). Hua *et al*. (2004) isolated *A. popoffi* from the urine of a patient with urinary tract infection (Hua *et al*., 2004). *A. salmonicida*, generally known to infect cold blooded animals, has also been isolated from blood sample of a patient in India (Tewari *et al*., 2014). *A. salmonicida* was identified by Vitek 2 compact automated system. Non-culturable *Aeromonas* can be found in drinking water in various concentrations. The first report of *Aeromonas* from drinking water was confirmed by sequencing 16S rRNA (Figueras *et al*., 2005). Different concentrations of *Aeromonas* have been detected in consumable products from markets (Isonhood and Drake, 2002).

Epidemiology

Mesophilic bacteria grow well at higher temperatures and therefore an increase in bacterial load may be attributed to their increase in concentration in both freshwater environments and drinking water sources with the increase of ambient temperature (Moyer, 1987; Edberg *et al*., 2007; Khardori and Fainstein, 1988). The seasonality is also seen in extra-intestinal infections such as septicemia, where 42% to 67% of bacteremic diseases appear during the summer season (Tsai *et al*., 2006). The elevated levels of these bacteria in aquatic environments during the summer season increases the opportunities of human or aquatic organisms of getting exposed to them and thus the risk of getting infected by these bacteria also gets higher. Infections caused by aeromonads seem to be rather more prevalent in developing countries like India, Bangladesh, Brazil, China, Cuba, Egypt, Iran, Libya, Nigeria, Venezuala and Vietnam (Ghenghesh *et al*., 2008). Prevalence of *Aeromonas* related disease is more during rainy seasons when the water salinity is low than at high salinity during dry season (Marcel *et al*., 2002).

Infections and Symptoms

Gastrointestinal tract is the most common site of *Aeromonas* infection. Evidences show that *Aeromonas*-associated diarrhoea or cholera-like disease occurs in some patients, whereas no symptom may appear in cases of low-level infections (Gurwith *et al*., 1977; Holmberg *et al*., 1984). Kelly *et al*. (1993) isolated *Aeromonas* from non-fecal samples from 58 patients, suffering from gangrene, septicemia, osteomyelitis and peritonitis. *Aeromonas*-related diarrhoea may be watery and self-limiting. In other cases, fever, abdominal pain and bloody diarrhea may develop along with dehydration (Ghenghesh *et al*., 1999). Hematologic cancer patients, patients with tumours in their gastrointestinal tract or having alimentary canal diseases are more likely to be infected by *Aeromonas*. In rare cases of segmental colitis *Aeromonas* segmental colitis may occur that seem to be ischemic colitis or Crohn's disease (Bayerdorffer *et al*., 1986). Although any portion of the colon may be affected, it mostly affects the ascending or transverse sections. Iileal ulceration has also been linked to *Aeromonas enteritis* (Yamamoto *et al.,* 2004). It may also cause intra-mural intestinal hemorrhage including small bowel obstruction (Block *et al*., 1994),

Table I
Genomospecies and phenospecies of the genus *Aeromonas*.

DNA Hybridization group	Type Strain/ Reference	Genospecies	Phenospecies	Remarks	Reference
1	ATCC 7966	*A. hydrophila*	*A. hydrophila*	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
1	BCCM/LMG 19562	*A. hydrophila* subsp. *dhakensis*	*A. hydrophila* subsp. *dhakensis*	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
1	BCCM/LMG 19707	*A. hydrophila* subsp. *ranae*	*A. hydrophila* subsp. *ranae*	Pathogenic for frogs	Martin-Carnahan and Joseph, 2005
2	ATCC 14715	*A.bestiarum*	*A. hydrophila- like*	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
3	ATCC 33658	*A. salmonicida*	*A. salmonicida* subsp. *Salmonicida*	Nonmotile fish pathogen	Martin-Carnahan and Joseph, 2005
3	ATCC 33659	*A. salmonicida*	*A. salmonicida* subsp. *Achromogenes*	Nonmotile fish pathogen	Martin-Carnahan and Joseph, 2005
3	ATCC 27013	*A. salmonicida*	*A. salmonicida* subsp. *Masoucida*	Nonmotile fish pathogen	Martin-Carnahan and Joseph, 2005
3	ATCC 49393	*A. salmonicida*	*A. salmonicida* subsp. *Smithia*	Nonmotile fish pathogen	Martin-Carnahan and Joseph, 2005
3	CDC 0434-84, Popoff C316	Unnamed	*A. hydrophila*- like	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
4	ATCC 15468	*A. caviae*	*A. caviae*	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
5A	CDC 0862-83	*A. media*	*A. caviae*-like	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
5B	CDC 0435-84	*A. media*	*A. media*	–	Martin-Carnahan and Joseph, 2005
6	ATCC 23309	*A. eucrenophila*	*A. eucrenophila*	–	Martin-Carnahan and Joseph, 2005
7	CIP 7433, NCMB 12065	*A. sobria*	*A. sobria*	–	Martin-Carnahan and Joseph, 2005
8X	CDC 0437-84	*A. veronii*	*A. sobria*	–	Martin-Carnahan and Joseph, 2005
8Y	ATCC 9071	*A. veronii*	*A. veronii* biovar sobria	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
9	ATCC 49568	*A. jandaei*	*A. jandaei*	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
10	ATCC 35624	*A. veronii* biovar *veronii*	*A. veronii* biovar *veronii*	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
11	ATCC 35941	Unnamed	*Aeromonas* spp. (ornithine Positive	–	Martin-Carnahan and Joseph, 2005
12	ATCC 43700	*A. schubertii*	*A. schubertii*	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
13	ATCC 43946	*Aeromonas* Group 501	*A. schubertii*-like	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
14	ATCC 49657	*A. trota*	*A. trota*	Isolated from clinical specimens	Martin-Carnahan and Joseph, 2005
15	ATCC 51208, CECT 4199	*A. allosaccharophila*	*A. allosaccharophila*	–	Martin-Carnahan and Joseph, 2005
16	ATCC 51020	*A. encheleia*	*A. encheleia*	Pathogenic for eels	Martin-Carnahan and Joseph, 2005
17	BCCM/LMG 1754	*A. popoffii*	*A. popoffii*	–	Martin-Carnahan and Joseph, 2005
UA	MTCC 3249, NCIM 5147	*A. culicicola*	*A. culicicola*	Isolated from mosquitoes	Martin-Carnahan and Joseph, 2005

Table I. Continued.

DNA Hybridization group	Type Strain/ Reference	Genospecies	Phenospecies	Remarks	Reference
UA	–	*A. eucrenophila*	*A. tecta*	Isolated from clinical and environmental sources	Demarta *et al.*, 2008
UA	–	*A. trota*	*A. aquariorum*	Isolated from monkey faeces	Harf-Monteil *et al.*, 2004
UA	–	*A. popoffii*	*A. bivalvium*	Isolated from aquaria of ornamental fish	Martinez-Murcia *et al.*, 2008
UA	–	Unnamed	*A. sharmana*	Isolated from bivalve molluscs	Minana-Galbis *et al.*, 2004
UA	868E^T (=CECT 7113^T =LMG 23376^T)	*A. bivalvium* sp. nov.	–	Isolated from bivalve molluscs	Minana-Galbis *et al.*, 2007
UA	–	*A. schubertii*	*A. simiae*	Isolated from midgut of Mosquitoes	Pidiyar *et al.*, 2002
UA	–	*A. sharmana* sp. nov.	*A. sobria*	Isolated from a warm spring	Saha and Chakrabarti, 2006
UA	266^T (5CECT 8023^T 5LMG 26707^T)	*Aeromonas australiensis* sp. nov.	*Aeromonas fluvialis*, *Aeromonas veronii* and *Aeromonas allosaccharophila*	Isolated from irrigation water system	Aravena-Roman *et al.*, 2013
UA	A.11/6T (=DSMZ 24095T, =CECT 7828T)	*Aeromonas lusitana* sp. nov.	–	Isolated frm untreated water and vegetables (lettuce/celery)	Martinez-Murcia *et al.*, 2016
UA	ATCC 49803	*Aeromonas enteropelogenes*	*Aeromonas trota*	Isolated from human stool	Schubert *et al.*, 1990
UA	CECT 4254T	*Aeromonas diversa*	*Aeromonas schubertii*	Isolated from leg wound of a patient	Farfan *et al.*, 2013
UA	717^T (=CECT 7401^T =LMG 24681^T)	*Aeromonas fluvialis*	*Aeromonas veronii*	Isolated from river water	Alperi *et al.*, 2010b
UA	848T^T (=CECT 5864^T =LMG 22214^T)	*Aeromonas molluscorum* sp. nov.	–	Isolated from Wedge-shells	Minana-Galbis *et al.*, 2004
UA	WB4.1-19^T (CECT 7518^T DSM 22539^T MDC 2511^T)	*Aeromonas rivuli* sp. nov.	–	Isolated from a karst hard water creek	Figueras *et al.*, 2011
UA	S1.2^T (=CECT 7443^T =LMG 24783^T)	*Aeromonas piscicola* sp. nov.	–	Isolated from wild diseased Salmon	Beaz-Hidalgo *et al.*, 2009
UA	A2-50^T (=CECT 7403^T =LMG 24683^T)	*Aeromonas taiwanensis* sp. nov.	–	Isolated from wound infection of a patient	Alperi *et al.*, 2010a
UA	A2-67^T (=CECT 7402^T =LMG 24682^T)	*Aeromonas sanarellii* sp. nov.	–	Isolated from a wound culture from a patient	Alperi and Figueras, 2010
UA		*A. hydrophila*	–	Isolated from wild birds	Glunder and Seigmann, 1989

UA-Unassigned; – Un Named

and refractory inflammatory bowel disease (Doman *et al.*, 1989). Gastrointestinal tract infection symptoms may mimic cholera (Mohan *et al.*, 2017).

The second most common area of *Aeromonas*-related infection in our body is the skin and the soft tissues underlying the skin. Aeromonads may cause several types of skin and soft tissue infections, ranging from mild problems like pustular lesions to dangerous conditions that can cause morbidity in infected person. Some of these conditions include cellulitis, necrotizing fasciitis, myonecrosis, septic arthritis and septic shock (Lai *et al.*, 2007). Some medical treatment procedures like medicinal leech therapy, appendectomies, colectomy, cholecystectomy and elective surgery enhance

the chances of *Aeromonas*-associated wound infections (Moawad and Zelderman, 2002; Tena *et al*., 2009). *A. hydrophila* and *A. caviae* were isolated from five burn patients admitted in Royal Brisbane hospital, where the patients had been immersed in water immediately after getting burnt, putatively contaminated with *Aeromonas* (Kienzle *et al*., 2000).

A. hydroplila sensu stricto, *A. caviae* and *A. veronii* bv. Sobria have been implicated in blood borne infections. Less frequently, three other species namely, *A. jandaei, A. veronii* bv. *veronii* and *A. schubertii* are known to cause sepsis (Janda *et al*., 1994). *Aeromonas*-septicemia is more prevalent in immunocompromised conditions viz. myeloproliferative disorders, chronic liver disease, neoplasia, biliary disease, AML, myeloplastic syndromes, non-Hodgkin's lymphoma and acute lymphocytic leukemia (Ko *et al.,* 2000; Tsai *et al*., 2006). *Aeromonas* septicemia is also related to diseases like diabetes mellitus, renal and cardiac problems, thallasemia, multiple myeloma, aplastic anemia and Waldenstrom's macroglobulinemia (Janda and Abbott, 1996; Padmaja *et al*., 2013). Aeromonad-contaminated catheters and dialysis chambers may serve as points of entry into human blood. *Aeromonas* cause peritonitis and cholangitis as intra-abdominal disease. *Aeromonas*-associated cholangitis may result in pancreatic carcinoma, cholangiocarcinoma, cholelithiasis patients or patients with non-malignant biliary disease by the invasion of the bacteria from the gastrointestinal tract to the biliary tract via surgery or endoscopy (Chan *et al*., 2000). *A. hydrophila*, *A. veronii* bv. Sobria, *A. popoffi* and *A. caviae* infections have been implicated in UTI, aspiration pneumonia, keratitis, endophthalmitis, corneal ulceration and blood stream infections through biofilm formation (Ender *et al*., 1996; Hsueh *et al*., 1998; Miyake *et al*., 2000; Hua *et al*., 2004; Pinna *et al*., 2004; Hondur *et al*., 2008; Tang *et al*., 2014). First case of neonatal meningitis in a premature baby has been reported recently caused by *A. hydrophila* (Kali *et al*., 2016).

Aeromonas affects both cold and warm-blooded non-human animals. Mass deaths in fishes occur every year due to *Aeromonas*-associated diseases resulting in huge economic loss to the fish industry (Monette *et al*., 2006). Furunculosis in the salmonids, caused by *A. salmonicida sensu stricto* is characterized by symptoms like heamorrhages at fin bases, muscles and internal organs; loss of appetite, disordered melanin production, loss of energy and exopthalmia (Austin, 1997). Secondly, septicemia in carps, tilapia, catfishes, salmons, cods, bass and freshwater prawns is caused by *A. hydrophila* and *A. veronii* (Joseph and Carnahan, 1994). *A. hydrophila* has been detected in tissues like kidney, liver and blood of carps in farms (Mohanty *et al*., 2008). Incidences of *A. hydrophila* seem to be more prevalent than *A. caviae* and *A. sobria*, which indicates that *A. hydrophila*, is more virulent than the other (Daood, 2012). In a very recent study it was shown that *A. caviae* infection causes thrombocytopenia which contributes to elongation of clotting time which leads to hamorrhages in internal organs, muscles and bases of fins (Baldissera *et al*., 2018). Diseases in other ectothermic animals include ulcers (Lizards and snakes), "red leg" disease (frogs), septicemia (dogs), septic arthritis (calves), vesiculitis (bulls) (Gosling, 1996).

Pathogenicity

The identified virulence factors in *Aeromonas* are haemolysins, cytotoxins, enterotoxins, proteases [serine protease (AspA), elastase (AhpB)], lipases (Pla and Plc, Sat), DNAses, adhesins [type IV pili, polar flagella (FlaA and FlaB)] (Agarwal *et al*., 1998; Cascon *et al*., 2000; Rabaan *et al*., 2001), capsule and T3SS (Grim *et al*., 2013). Genome sequencing and annotation can be used to detect these virulence factors in *Aeromonas* (Grim *et al*., 2013). Enterotoxins, Act and Ast (Sha *et al*., 2002), elastase (Cascon *et al*., 2000), flagellin (Rabaan *et al*., 2001), and Stx1 and Stx2 (Alperi and Figueras, 2010) are directly involved in the pathogenesis. In a study *Aeromonas* isolates from well, tap and bottled water samples were found to have *aer* and *ast* genes, which poses a serious health concern for the human society (Didugu *et al*., 2015).

Aeromonas inections are mostly polymicrobial (Figueras and Beaz-Hidalgo, 2015), in which there is competition and cooperation between the bacterial cells (Armbruster *et al*., 2016). Virulence when checked in *C. elegans* was found to be higher in paired *Aeromonas* infections than in single strain (Mosser *et al*., 2015). The dual strain *A. hydrophila* infection showed synergistic effect by local tissue damage and antagonistic effect by elimination (Ponnusamy *et al*., 2016). The pathogenic potential of *A. veronii* isolates from clinical samples when tested were found to be like the drinking water and environmental isolates (Lye, 2011). The protein secretion systems of *Aeromonas* play important roles in pathogenesis caused by them. The type II secretion system is associated with the extracellular release of proteases, amylases, DNases and aerolysin (Pang *et al*., 2015). Type III secretion system, which is found in greater frequency in clinical isolates than environmental ones (Pang *et al*., 2015) functions by inserting effective toxins inside the host cells (Sierra *et al*., 2010). The type VI secretion system allows insertion of virulence factors into host cells through valine-glycine repeat protein and hemolysin-coregulated proteins. These proteins when secreted show antimicrobial pore-forming properties or remain as structural proteins (Bingle *et al*., 2008).

Gastroenteritis. Aeromonads enter the human gut via oral cavity, escape the effects of gastric acidity and produce bacteriocin-like compounds, which facilitate colonization of the intestine. They attach themselves to gastrointestinal epithelium, form biofilm, colonize and elaborate virulence factors to cause infection. Bacterial flagella and pili play important roles in gastric pathogenicity (Kirov *et al*., 2000).

Wound infections. Virulence caused by *Aeromonas* and the virulence factors possessed by them are similar to those of Gram-negative *P. aeruginosa*. The first step is settlement of the bacteria in wound site with the help of adhesion factors such as OmpA protein (Namba *et al*., 2008). The second step involves production of proteases (metalloproteases, serine proteases and aminopeptidases) and the breakdown of proteinaceous material of the host cells to gain energy, for multiplication of bacilli (Janda, 2001). The third step includes the entry of aeromonads into deeper tissues via chemotactic motility (Janda, 1985).

Septicemia. Most cases of primary *Aeromonas* septicaemia apparently arise through transfer of bacteria from the gastrointestinal tract into the blood circulatory system. They may also travel to the bloodstream from infected wounds, peritonitis, or biliary disease. Most of the *Aeromonas* septicemias are caused by a small number of species. Specific strains having certain markers are only responsible for most of the blood-borne diseases. Aeromonads of sergroups O:11, O:16, O:18, and O:34 are responsible for most cases of septicemia, which shows that lipo-polysaccharide (LPS) antigens are important in causing systemic diseases. The presence of LPS or the S layers makes most *Aeromonas* isolates resistant to the lytic effects of the host's classical complement pathway (Janda *et al.,* 1994).

Genes involved in virulence

Cytotoxic enterotoxin (*act*), haemolysin (*hlyA*)/ aerolysin (*aerA*). The *act* gene of *A. hydrophila* encodes cytotoxic enterotoxin, which has many functions viz., cytotoxic, haemolytic and enterotoxic activities (Chopra and Houston, 1999). Other aeromonads have haemolytic activities due to the presence of other genes, namely *hlyA* and *aerA*, and these strains may have one or more of these genes (Heuzenroeder *et al*., 1999). The mature aerolysin binds to host cells, aggregates there and forms holes in their cell membrane destroying the permeability barrier of the membrane, which ultimately leads to osmotic lysis of the cells (Howard and Buckley, 1982). The haemolysin induces accumulation of fluids in intestinal loops (Asao *et al*., 1986), release of certain inflammation promoting factors from the granulocytes (Scheffer *et al*., 1988) and apoptosis of the host cells (Nelson *et al*., 1999). A study showed that about 50% of the marine fish samples were positive for the haemolysin gene *hyl* in India (Reshma *et al*., 2015). In another study, both environmental and clinical isolates from Kolkata (erstwhile Calcutta) in India were found to be positive for *act* and the enteropathogenic potential of these isolates were found to be comparable to *V. cholerae* (Bhowmik *et al*., 2009).

Cytotonic enterotoxins (*ast*, *alt*). The cytotonic enterotoxins do not degenerate the small intestine. The clones of *E. coli* having cytotonic enterotoxin genes have been showed to cause elongation of Chinese hamster ovary (CHO) cells, which also produces cyclic AMP, and these are enterotoxic responses. The Alt enterotoxin is heat labile, whereas Ast is heat stable at 56°C (Chopra and Houston, 1999). These genes have strong roles in causing diarrhoea (Sha *et al*., 2002).

Elastase (*ahpB*). The knocking out of the *ahpB* gene in *A. hydrophila* causes a high rise in the LD_{50} value of *A. hydrophila* in fishes, which indicates that elastase, a zinc metalloprotease, is an important virulence factor to cause disease in organisms (Cascon *et al*., 2000). The *ahpB* gene in *A. hydrophila* encodes protease with both elastolytic and caseinolytic activities (Cascon *et al*., 2000).

Flagella. Most of the *Aeromonas* species and all of the species responsible for human pathogenesis are motile having polar flagella. The polar flagellum has five flagellin subunits *Fla A*, *Fla B*, *Fla G*, *Fla H* and *Fla J*. The *flaA* and *flaB* genes have been cloned and sequenced from *A. salmonicida* (Umelo and Trust, 1997). All the five genes (*flaA*, *flaB*, *flaG*, *flaH* and *flaJ*) were identified in polar flagellin locus of *A. caviae.* Motility is known as an important virulence factor in the aeromonads. Mutation in either *flaA* or *flaB* did not affect development of flagellum but did reduce adherence and motility by approximately 50%. Mutations in *flaH*, *flaJ* or both cause complete loss of motility, development of flagellum and ability to get attached to HEp-2 cells. Thus, the ability to get attached to Hep-2 cells depends on motility and presence of flagella of aeromonads (Rabaan *et al*., 2001).

Lipase. Lipases change the plasma membrane of the host, increasing the severity of disease (Nawaz *et al.,* 2010). Lipase gene has been recovered from multidrug-resistant virulent aeromonads capable of forming biofilms isolated from cattle feaces (Igbinosa *et al*., 2015).

Shiga toxins (*Stx1 and Stx2*). Shiga toxins are protein toxins, which have two parts A and B. One part has enzymatic property and the other binds to the surface of the host cells. These toxins inhibit protein synthesis of the host cells (Sandvig, 2001) and also induce apoptosis (Jones *et al*., 2000).

Enolase. Enolase is a glycolytic enzyme expressed in cell surfaces, which binds to human plasminogen

leading to the production of plasmin which degrade plasma proteins. Enolase is also a heat-shock protein, which regulated transcription and is also necessary for cell viability (Sha *et al*., 2009).

Others. Other virulence factors include adhesins (Huang *et al*., 2015), nucleases (Ji *et al*., 2015), pore forming toxins (Saurez *et al*., 2012) and catalysts.

Antimicrobial Susceptibility

All species of *Aeromonas* show similar antibiotic susceptibility profiles, which are also independent of the origin of the isolates (Kampfer *et al*., 1999). Most of the aeromonads have inducible chromosomal lactamases, which are their main resistance mechanisms. Among these, metallo-β-lactamases, which work against carbapenems, are of major concern (Janda, 2001; Zhiyong *et al.*, 2002). The Clinical and Laboratory Standards Institute (CLSI) have published consensus guideline for testing *Aeromonas* (Jorgensen and Hindler, 2007). The susceptibility status of *Aeromonas* isolates for therapeutically active drugs also seem to be species independent with one exception of *Aeromonas trota*, which is susceptible to ampicillin (Carnahan *et al*., 1991). In a study antibiotic resistance status of *Aeromonas* isolates from diseased fishes were found to be similar to those isolated from the freshwater fish farm (Daood, 2012). In another study *Aeromonas* strains resistant to mercury and arsenite were found and these got transferred to *E. coli* when conjugation experiments were performed (Huddleston *et al.*, 2006).

Resistance Mechanisms. Three major classes of β-lactamases are present in *Aeromonas* species, viz, C cephalosporinase, D penicillinase, and a class B metallo-β-lactamase (MBL) (Libisch *et al*., 2008). Fosse *et al.* (2003) classified strains expressing these β-lactamases into five groups as *A. hydrophila*: class B, C, and D β-lactamases, *A. caviae*: class C and D β-lactamases, *A. veronii*: class B and D lactamases, *A. schubertii*: class D lactamases and *A. trota:* class C β-lactamases. Many *A. veronii* bv. Sobria isolates also express a class C cephalosporinase. In few cases, infecting *Aeromonas* strains expressed a class A β-lactamase of the TEM family of ESBLs (Extended Spectrum β-Lactamases), a character similar to the *Enterobacteriaceae* (Marchandin *et al*., 2003). The β-lactamases are involved in detoxification of antibiotics, changes in the drug binding site of the target and inhibiting the entry of the drug into the bacterial cells by causing changes in structure and function of the cytoplasmic and cell membranes (Benveniste and Davies, 1973). Each strain can produce a maximum of three β-lactamases, which work in a coordinated manner (Walsh *et al.*, 1997). Class C cephalosporinases of the AmpC family are resistant to cephamycins, extended spectrum cephalosporins and β-lactamase inhibitor compounds, like clavulanic acid, tazobactam, and sulbactam, which hydrolyse the CO-NH bond in the lactum ring of cephalosporin to inactivate it (Fosse *et al.*, 2003).

"CphA", is the most common MBL produced by *Aeromonas* species, which is largely found in *A. hydrophila* and *A. veronii* isolates (Walsh *et al.,* 1997). Two other MBLs (VIM and IMP) are also found in *A. hydrophila* and *A. caviae* strains, which encode an integron and a plasmid, respectively (Libisch *et al*., 2008). These MBL-producing strains are resistant to ceftazidime, cefepime, imipenem, and piperacillin-tazobactam; both strains are found to be susceptible to aztreonam *in vitro*. MBLs work in a two-step process: firstly, the C-N bond of the beta-lactam antibiotic is cleaved and then the binding nitrogen is protonated (Crowder *et al*., 2006).

Recently, NDM-1 ($bla_{\text{NDM-1}}$) gene has been detected in this group of bacteria (Walsh *et al.*, 2011). The spread of mobile NDM-1, also known as carbapenemase, is of great concern, not only because these enzymes confer resistance to carbapenems and other β-lactam antibiotics, but also because such pathogens typically are resistant to multiple antibiotic classes, making treatment difficult. Plasmids having the sequence encoding this carbapenemase can have up to 14 other antibiotic-resistance determinants and can make other bacteria also resistant, resulting in multi-drug resistant or extreme drug-resistant phenotypes. Resistance of this scale could have serious public health implications because modern medicine is dependent on the ability to treat infection (Livermore, 2009).

Quinolone resistance in *Aeromonas* strains isolated from two European rivers is a matter of rising concern because quinolone was previously known to be effective in combating *Aeromonas* infections (Goni-Urriza *et al*., 2000). Several *A. caviae* strains showed resistance to nalidixic acid, ciprofloxacin, and norfloxacin (Sinha *et al.*, 2004). Aeromonads pathogenic to fish are found to be resistant to amoxicillin, ampicillin-sulbactum and streptomycin (Abu-Elala *et al*., 2015). These antibiotic resistant bacteria come into the environment through improper septic systems, agriculture and wastewater treatment plants (Rosenblatt-Farrell, 2009). River sediments adsorb antibiotics (Zhou *et al*., 2011) some of which may remain there for months (Lai *et al*., 2011). These impart antibiotic resistance to bacterial populations at that location. Biofilm formation increases resistance to antimicrobial substances (Acker *et al*., 2014), disinfectants (Jahid and Ha, 2014). Biofilm formation in *Aeromonas* is affected differently in different strains under several food related stresses. However, low temperature and pH conditions were found to facilitate biofilm formation in a recent study, which is the first study of this kind regarding *Aeromonas* (Nagar *et al*., 2017).

Role of plamids, integron systems and transposons in disease transmission

In *Aeromonas*, gene transfer mainly occurs through conjugation and transformation, in which type IV pili play a vital role (Huddleston *et al*., 2013). In a study, seven ESBL and two AmpCBL-producing *Aeromonas* strains were able to transfer their antibiotic resistance genes to *E. coli* (Bhaskar *et al*., 2015). Bacterial conjugative plasmids, transposable elements and integron systems are the panoply on which bacteria depend for their resistance to anti-bacterial compounds. Plasmids in particular serve as a platform on which useful resistance genes are assembled and subsequently disseminated (Bennett, 2008). Plasmid profiling and molecular characterization of aeromonad plasmids were undertaken by several research groups to address to the problems of generation and transmission of antibiotic resistance genes (Toranzo *et al*., 1983; Rhodes *et al*., 2000). Studies in eastern India focussed on the characterization of *Aeromonas* spp. isolated from cyprinid and silurid fishes affected with ulcerative disease (EUS) and the involvement of a low molecular weight plasmid has been implicated in the etiology of this disease in fishes (Pradhan and Pal, 1990; Majumdar *et al*., 2006; Majumdar *et al*., 2007). Subsequent investigations have also proved that, the degree of antibiotic resistance in these bacterial isolates is gradually increasing through the years (Pradhan and Pal, 1993; Saha and Pal, 2002; Das *et al*., 2009; Pal and Bhattacharjee, 2011).

Our laboratory tested antibiotic resistance status in few environmental *Aeromonas* isolates and the results showed an increase in antibiotic resistance in case of some antibiotics, while decrease in resistance in others (Dey Bhowmick and Bhattacharjee, 2017). Since antibiotic resistance is increasing in *Aeromonas,* aquaculture should resort to alternate means such as probiotics, essential oils and phage therapy to combat this problem.

In contrast to bacterial conjugative plasmids, which tend to be larger, mobilizable resistance plasmids tend to be relatively smaller (~10 to 20 kb) and encode only a handful of genes including the resistance gene(s) (Bennet, 2008). Therefore, resistance to multi-drugs and presence of small-sized plasmids in environmental isolates of this medically important bacteria group may indicate potential threat to human and culture fisheries (Pal and Bhattacharjee, 2011). Through horizontal gene transfer R-plasmids are spread between different species of *Aeromonas*, which spread multi-drug resistance (Indra *et al*., 2015). Transfer of antibiotic resistance genes from *Aeromonas* to other environmental and clinical bacteria makes treatment of both fish and humans difficult. Presence of multidrug resistance genes on mobile genetic elements is therefore a serious threat to society (Piotrowska and Popowska, 2015).

Conclusion

Phenotypically and genotypically a heterogenous group, aeromonads have been detected, isolated and characterized from varied sources such as brackish, fresh, estuarine, marine waters, chlorinated and unchlorinated water supplies, heavily polluted waters, cold and warm-blooded animals and humans alike. In contrast to the traditional morphological and biochemical differentiation, identification of aeromonads from clinical and environmental sources are presently based on PCR-based genotyping approach such as ribotyping and analysis of gyrB.

In the post World War II period, extensive use (or abuse) of antibiotics have given rise to drug-resistant varieties of bacteria, owing to the success and speed of bacterial adaptation. Bacteria apply many mechanisms to show antibiotic resistance. These resistance genes get accumulated in plasmids and are thought to spread among other bacteria through them. In order to find solution to this problem many researchers have undertaken plasmid profiling and molecular characterization of aeromonad plasmids (Toranzo *et al.,* 1983). Therefore, assessment of anti-microbial drug resistance and possible involvement of bacterial plasmids in this resistance, in the locally isolated clinically and agriculturally important aeromonads, may be rewarding. To understand fully the virulence potential of any pathogen, it is imperative to understand pathogenic factors and/or mechanisms that are involved in their virulence. This is crucial since the expression of different virulence genes could contribute to infection depending upon the anatomical niche where the pathogenic organisms colonize and the microenvironment that dictates the differential expression of genes.

So far, many virulence factors have been discovered and characterized from *Aeromonas* group, especially from *A. hydrophila*, the causative organism of septicemia, wound infections and diarrhoea in humans and in animals. Novel putative virulence factors and/or virulence transfer systems, such as the NDM-1 gene, are being discovered on a regular basis in this diverse and ubiquitous group of bacteria. Although its emergence and distribution is controversial, the detection of NDM-1 gene in this clinically and agriculturally important bacteria group calls for a detailed surveillance of antibiotic resistance and also mode of transferability of NDM-1 gene in this bacteria group.

Plasmid-mediated horizontal gene transfer and acquisitions are thought to be one of the many adaptive ways by which bacteria acquire genes that may be useful periodically in combating environmental stresses, *e.g.*, confronting potentially hazardous anti-bacterial agents, such as antibiotics (Bennett, 2008). Useful genes are thought to be selected and persist that ultimately confer

better adaptability to microorganisms. Plasmid profiling in pathogenic isolates of *A. hydrophila* from fishes with ulcers, had been done to investigate plasmid-mediated virulence potential of the bacterium. Plasmid profiling, plasmid-mediated antibiotic resistance and pathogenesis in aeromonads have been investigated by several groups but further works are necessary to investigate the mode of transmission of virulence and drug-resistance genes in this bacterial group. This is imperative in heavily populated tropical countries like India, especially where sanitary requirements are not upto the standard.

Moreover, knowledge on how antibiotic resistance develops and is spread by mobile genetic elements is necessary for designing and developing prevention strategies intended to minimize the threat of bacterial infections. Considering the great adaptive ability of these bacteria vis-a-vis the environmental stresses and increasing use of anti-bacterial agents in combating *Aeromonas*-associated pathogenesis, newer virulence genes may be acquired by these organisms. Therefore, a search and mapping for putative virulence genes of *Aeromonas* should be undertaken.

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Literature

Abeyta C.Jr., C.A. Kaysner, M.M. Wekell, J.J. Sullivan and G.N. Stelma. 1986. Recovery of *Aeromonas hydrophila* from oysters implicated in an outbreak of food borne illness. *J. Food Prot*. 49: 643–646.

Abu-Elala N., M. Abdelsalam, Sh. Marouf and A. Setta. 2015. Comparative analysis of virulence genes, antibiotic resistance and gyrB-based phylogeny of motile *Aeromonas* species isolates from Nile tilapia and domestic fowl. *Lett. Appl. Microbiol.* 61: 429–436.

Acker H.V., P.V. Dijck and T. Coenye. 2014. Molecular mechanisms of antimicrobial tolerance and resistance in bacterial and fungal biofilms. *Trends. Microbiol*. 22: 326–333.

Agarwal R.K., K.N. Kapoor and A.J. Kumar. 1998. Virulence factors of aeromonads-an emerging food borne pathogen problem. *A. J. Commun. Dis.* 30: 71–78.

Alperi A. and M.J. Figueras. 2010. Human isolates of *Aeromonas* possess shiga toxin genes (*stx1* and *stx2*) highly similar to the most virulent gene variants of *Escherichia coli*. *Clin. Microbiol. Infect.* 16: 1563–1567.

Alperi A., A.J. Martinez-Murcia, A. Monera, M.J. Saavedra and M.J. Figueras. 2010b. *Aeromonas fluvialis* sp. nov., isolated from a Spanish river. *Int. J. Syst. Evol. Micobiol.* 60: 72–77.

Alperi A., A.J. Martinez-Murcia, W.C. Ko, A. Monera, M.J. Saavedra and M.J. Figueras. 2010a. *Aeromonas taiwanensis* sp. nov. and *Aeromonas sanarellii* sp. nov., clinical species from Taiwan. *Int. J. Syst. Evol. Microbiol.* 60: 2048–2055.

Altwegg M., G. Martinetti Lucchini, J. Luthy-Hottenstein and M. Rohrbach. 1990. *Aeromonas*-associated gastroenteritis after consumption of contaminated shrimp. *Eur. J. Clin. Microbiol. Infect. Dis.* 10: 44–45.

Anguita J., L.B.R. Aparicio and G. Naharro. 1993. Purification, gene cloning, amino acid sequence analysis and expression of an extracellular lipase from an *Aeromonas* hydrophila human isolate. *Appl. Environ. Microbiol.* 59: 2411–2417.

Aravena-Roman M., R. Hidalgo, T.J.J. Inglis, T.V. Riley, A.J. Martinez-Murcia, B.J. Chang and M.J. Figueras. 2013. *Aeromonas australiensis* sp. nov., isolated from irrigation water. *Int. J. Sys. Evol. Microbiol.* 63: 2270–2276.

Armbruster C.A., D.J. Wolter, M. Mishra, H.S. Hayden, M.C. Radey, G. Merrihew, M.J. MacCoss, J. Burns, D.J. Wozniak, M.R. Parsek and others. 2016. *Staphylococcus aureus* Protein A mediates interspecies interactions at the cell surface of *Pseudomonas aeruginosa. mBio.* 7: 00538–16.

Asao T., S. Kozaki, K. Kato, Y. Kinoshita, K. Otsu, T. Uemura, and G. Sakaguchi. 1986. Purification and characterization of an *Aeromonas hydrophila* hemolysin. *J. Clin. Microbiol*. 24: 228–232.

Austin B. 1997. Progress in understanding the fish pathogen *Aeromonas salmonicida*. *Mar. Biotechnol.* 15: 131–134.

Baldissera M.D., C.F. Souza, C.M. Verdi, B.S. Vizzotto, R.C.V. Santos and B. Baldisserotto. 2018. *Aeromonas caviae* alters the activities of ecto-enzymes that hydrolyze adenine neucleotides in fish thrombocytes. *Microb. Pathog*. 115: 64–67.

Bayerdorffer E., G. Schwarzkopf-Steinhauser and R. Ottenjann. 1986. New unusual forms of colitis: report of four cases with known and unknown etiology. *Hepatogastroenterology* 33: 187–190.

Beaz-Hidalgo R., A. Alperi, M.J. Figueras and J.L. Romalde. 2009. *Aeromonas piscicola* sp. nov., isolated from diseased fish. *Syst. Appl. Microbiol.* 32: 471–479.

Bennett P.M. 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br. J. Pharmacol.* 153: S347–S357.

Benveniste R. and J. Davies. 1973. Mechanims of antibiotic resistance in bacteria. *Annu. Rev. Biochem*. 42: 471–506.

Bhaskar M., K.P. Dinoop and J. Mandal. 2015. Characterization of ceftriaxone-resistant *Aeromonas* spp. isolates from stool samples of both children and adults in Southern India. *J. Health Popul. Nutr*. 33: 1–5.

Dey Bhowmick U. and S. Bhattachrjee. 2017. Status of antibiotic resistance in the Aeromonads of North Bengal with a special reference to change in resistance pattern through altitudinal gradient. *NBU J. Anim. Sc*. 11: 51–59.

Bhowmik P., P.K. Bag, T.K. Hajra, R. De, P. Sarkar and T. Ramamurthy. 2009. Pathogenic potential of *Aeromonas hydrophila* isolated from surface waters in Kolkata, India. *J. Med. Microbiol*. 58: 1549–1558.

Bingle L.E.H., C.M. Bailey and M.J. Pallen. 2008. Type VI secretion: a beginner's guide. *Curr. Opin. Microbiol.* 11: 3–8.

Block K., J.M. Braver and F.A. Farraye. 1994. *Aeromonas* infection and intramural hemorrhage as a cause of small bowel obstruction. *Am. J. Gastroenterol.* 89: 1902–1903.

Carnahan A.M., T. Chakraborty, G.R. Fanning, D. Verma, A. Ali, J.M. Janda and S.W. Joseph. 1991. *Aeromonas trota* sp. nov., an ampicilin-susceptible species isolated from clinical specimens. *J. Clin. Microbiol.* 29: 1206–1210.

Cascon A., J. Yugueros, Temprano, A. Temprano, M. Sanchez, C. Hernanz, J.M. Luengo and G. Naharro. 2000. A major secreted

elastase is essential for pathogenicity of *Aeromonas hydrophila*. *Infect. Immun*. 68: 3233–3241.

Chakraborty T., B. Huhle, H. Bergbaur and W. Goebel. 1986. Cloning, expression, and mapping of the *Aeromonas hydrophila* aerolysin gene determinant in *Escherichia coli* K-12. *J. Bacteriol.* 167: 368–374.

Chan F.K.L., J.Y.L. Ching, T.K.W. Ling, S.C.S. Chung and J.J.Y. Sung. 2000. *Aeromonas* infection in acute suppurative cholangitis: review of 30 cases. *J. Infect*. 40: 69–73.

Chopra A.K. and C.W. Houston. 1999. Enterotoxins in *Aeromonas*-associated gastroenteritis. *Microb. Infect.* 1: 1129–1137.

Chopra A.K., C.W. Houston, J.W. Peterson and G.F. Jin. 1993. Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. *Can. J. Microbiol.* 39: 513–23.

Crowder M.W., J. Spencer and A.J. Vila. 2006. Metallo-lactamases: Novel weaponry for antibiotic resistance in bacteria. *Acc. Chem. Res*. 39: 721–728.

Daood N. 2012. Isolation and antibiotic susceptibility of *Aeromonas* spp. from freshwater fish farm and farmed carp (Dam of 16 Tishreen, Lattakia). *Damascus Univ. J. Basic Sci*. 28: 27–39.

Das A., D. Saha and J. Pal. 2009. Antimicrobial resistance and *in vitro* gene transfer in bacteria Isolated from ulcers of EUS-affected fishes in India. *Lett. Appl. Microbiol.* 49: 497–502.

Demarta A., M. Kupfer, P. Riegel, C. Harf-Monteil, M. Tonolla, R. Peduzzi, A. Monera, M.J. Saavedra and A. Martinez-Murcia. 2008. *Aeromonas tecta* sp. nov., isolated from clinical and environmental sources. *Syst. Appl. Microbiol.* 3: 278–286.

Didugu H., M. Thirtham, K. Nelapati, K.K. Reddy, B.S. Kumbhar, A. Poluru and G. Pothanaboyina. 2015. A study on the prevalence of *Aeromonas* spp. and its enterotoxin genes in samples of well water, tap water, and bottled water. *Vet. World* 8: 1237–1242.

Doman D.B., M.I. Golding, H.J. Goldberg and R.B. Doyle. 1989. *Aeromonas hydrophila* colitis presenting as medically inflammatory bowel disease. *Am. J. Gastroenterol.* 84: 83–85.

Edberg S.C., F.A. Browne and M.J. Allen. 2007. Issues for microbial regulation: *Aeromonas* as a model. *Crit. Rev. Microbiol.* 33: 89–100.

Ender P.T., M.J. Dolan, D. Dolan, J.C. Farmer and G.P. Melcher. 1996. Near-drowning-associated *Aeromonas* pneumonia. *J. Emerg. Med*. 14: 737–741.

Erova T.E., V.G. Kosykh, A.A. Fadl, J. Sha, A.J. Horneman and A.K. Chopra. 2008. Cold Shock Exoribonuclease R (VacB) Is Involved in *Aeromonas hydrophila* Pathogenesis. *J. Bacteriol.* 190: 3467–3474.

Farfan M., N. Spataro, A. Sanglas, V. Albarral, J.G. Loren, E. Bosch and M.C. Fustea. 2013. Draft Genome Sequence of the *Aeromonas diversa* Type Strain. *Genome Announc.* 1: 1–2.

Figueras M.J. and R. Beaz-Hidalgo. 2015. *Aeromonas* infections in humans, pp. 65–68. In J. Graf (ed.), *Aeromonas*. Norfolk Caister Academic Press.

Figueras M.J., A. Alperi, R. Beaz-Hidalgo, E. Stackebrandt, E. Brambilla, A. Monera and A.J. Martínez-Murcia. 2011. *Aeromonas rivuli* sp. nov., isolated from the upstream region of a karst water rivulet. *Int. J. Syst. Evol. Microbiol.* 61: 242–248.

Figueras M.J., A. Saurez-Franquet, M.R. Chacon, L. Soler, M. Navarro, C. Alejandre, B. Grasa, A.J. Martinez-Murcia and J. Guarro. 2005. First record of the rare species *Aeromonas culicicola* from a drinking water supply. *Appl. Environ. Microbiol.* 71: 538–541.

Figueras M.J., F. Latif-Eugenin, F. Ballester, I. Pujol, D. Tena, K. Berg, M.J. Hossain, R. Beaz-Hidalgo and M.R. Liles. 2017. '*Aeromonas intestinalis*' and '*Aeromonas enterica*' isolated from human faeces, '*Aeromonas crassostreae*' from oyster and '*Aeromonas aquatilis*' isolated from lake water represent novel species. *New Microbes New Infect*. 15: 74–76.

Fiorentini C., E. Barbieri, L. Falzano, W. Baffone, A. Pianetti, M. Katouli, I. Kuhn, R. Mollby, F. Bruscolini, A. Casiere and others. 1998. Occurrence, diversity and pathogenicity of mesophilic *Aeromonas* in estuarine waters of the Italian coast of the Adriatic Sea. *J. Appl. Microbiol*. 85: 501–511.

Fosse T., C. Giraud-Morin, I. Madinier and R. Labia. 2003. Sequence analysis and biochemical characterization of chromosomal CAV-1 (*Aeromonas caviae*), the parental cephalosporinase of plasmid-mediated AmpC 'FOX' cluster. *FEMS Microbiol. Lett*. 222: 93–98.

Ghenghesh K.S., S.F. Ahmed, R.A. Ei-Khalek, A. Ai-Gendy and J. Klena. 2008. *Aeromonas* Associated Infections in Developing Countries. *J. Infect. Dev. Ctries.* 2: 81–98.

Ghenghesh K.S., F. Bara, B. Bukris, A. El-Surmani and S.S. Abeid. 1999. Characterization of virulence factors of *Aeromonas* isolated from children with and without diarrhoea in Tripoli, Libya. *J. Diarrhoeal Dis. Res.* 17: 75–80.

Goni-Urizza M., L. Pineau, M. Capdepuy, C. Roques, P. Caumette and C. Quentin. 2000. Antimicrobial resistance of mesophilic *Aeromonas* spp. isolated from two European rivers. *J. Antimicrob. Chemother*. 46: 297–301.

Gosling P.J. 1996. *Aeromonas* species in disease of animals, p. 175–195. In B. Austin, M. Altwegg, P. J. Gosling, and S. Joseph (ed.), The genus *Aeromonas*. John Wiley & Sons Ltd., West Sussex, England.

Grim C.J., E.V. Kozlova, J. Sha, E.C. Fitts, C.J.V. Lier, M.L. Kirtley, S.J. Joseph, T.D. Read, E.M. Burd, B.D. Tall and others. 2013. Characterization of *Aeromonas hydrophila* Wound Pathotypes by Comparative Genomic and Functional Analyses of Virulence Genes. *mBio*. 4: 00064–13.

Gurwith M., C. Bourque, E. Cameron, G. Forrest and M. Green. 1977. Cholera-like diarrhea in Canada. Report of a case associated with enterotoxigenic *Escherichia coli* and a toxin producing *Aeromonas hydroplila. Arch. Intern. Med.* 137: 1461–1464.

Harf-Monteil C., A. Le Fleche, P. Riegel, G. Prevost, D. Bermond, P.A.D. Grimont and H. Monteil. 2004. *Aeromonas simiae* sp. nov., isolated from monkey faeces. *Int. J. Syst. Evol. Microbiol.* 54: 481–485.

Heuzenroeder M.W., C.Y.F. Wong and R.L.P. Flower. 1999. Distribution of two hemolytic toxin genes in clinical and environmental isolates of *Aeromonas* spp.: correlation with virulence in a suckling mouse model. *FEMS Microbiol. Lett.* 174: 131–136.

Holmberg S.D. and J.J. III. Farmer. 1984. *Aeromonas hydrophila* and *Plesiomonas shigelloides* as causes of intestinal infection. *Rev. Infect. Dis*. 6: 633–639.

Hondur A., K. Bilgihan, Clark, M.Y. Clark, O. Dogan, A. Erdinc and B. Hasanreisoglu. 2008. Microbiologic study of soft contact lenses after laser subepithelial keratectomy for myopia. *Eye Contact Lens.* 34: 24–27.

Howard S.P. and J.T. Buckley. 1982. Membrane glycoprotein receptor and hole-forming properties of a cytolytic protein toxin. *Biochemistry* 21: 1662–1667.

Hsueh P.R., L.J. Teng, L.N. Lee, P.C. Yang, Y.C. Chen, S.W. Ho and K.T. Luh. 1998. Indwelling device-related and recurrent infections due to *Aeromonas species. Clin. Infect. Dis.* 26: 651–658.

Hua H.T., C. Bollet, S. Tercian, M. Drancourt and D. Raoult. 2004. *Aeromonas popoffii* urinary tract infection. *J. Clin. Microbiol.* 42: 5427–5428.

Huang L., Y. Qin, Q. Yan, G. Lin, L. Huang, B. Huang and W. Huang. 2015. MinD plays an important role in *Aeromonas hydrophila* adherence to *Anguilla japonica* mucus. *Gene* 565: 275–281.

Huddleston J.R., J.M. Brokaw, J.C. Zak and R.M. Jeter. 2013. Natural transformation as a mechanism of horizontal gene transfer among environmental *Aeromonas* species. *Syst. Appl. Microbiol*. 36: 224–234.

Huddleston J.R., J.C. Zak and R.M. Jeter. 2006. Antimicrobial susceptibilities of *Aeromonas* spp. isolated from environmental sources. *Appl. Environ. Microbiol*. 72: 7036–7042.

Igbinosa I.H., E.O. Igbinosa and A.I. Okoh. 2015. Detection of antibiotic resistance, virulence gene determinants and biofilm formation in *Aeromonas* species isolated from cattle. *Environ. Sci. Pollut. Res.* 22: 17596–17605.

Indra U., M. Sureshkumar, B.L. Kumar and G. Vivekanandhan. 2015. Virulence determinants, drug and metal resistance of clinical and environmental *Aeromonas* species. *Int. J. Adv. Res.* 3: 573–587.

Isonhood J.H. and M. Drake. 2002. *Aeromonas* species in foods. *J. Food Prot*. 65: 575–582.

Jahid I.K. and S.D. Ha. 2014. Inactivation Kinetics of Various Chemical Disinfectants on *Aeromonas hydrophila* Planktonic Cells and Biofilms. *Foodborne Pathog. Dis*. 0: 1–8.

Janda J.M. 1985. Biochemical and exoenzymatic properties of *Aeromonas species. Diagn. Microbiol. Infect. Dis*. 3: 223–232.

Janda J.M. 2001. *Aeromonas* and *Plesiomonas,* pp. 1237–1270. In M. Sussman (ed.), Molecular medical microbiology, vol. 2. Academic Press, London, United Kingdom. Chapter 59.

Janda J.M. and P.S. Duffey. 1988. Mesophilic aeromonads in human disease: current taxonomy, laboratory identification, and infectious disease spectrum. *Rev. Infect. Dis.* 10: 980–987.

Janda J.M. and S.L. Abbott. 1996. Human pathogens, pp. 151–173. In: Austin B. *et al.* (eds). The genus *Aeromonas*. Wiley, London.

Janda J.M. and S.L. Abbott. 1998. Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentation, and unanswered questions. *Clin. Infect. Dis.* 27: 332–344.

Janda J.M., L.S. Guthertz, R.P. Kokka and T. Shimada. 1994. *Aeromonas species* in septicemia: laboratory characteristics and clinical observations. *Clin. Infect. Dis.* 19:77–83.

Jangid K., R. Kong, M.S. Patole and Y.S. Shouche. 2007. *luxRI* homologs are universally present in the genus *Aeromonas. BMC Microbiol.* 7: 93.

Ji Y., J. Li, Z. Qin, A. Li, Z. Gu, X. Liu, L. Lin and Y. Zhou. 2015. Contribution of nuclease to the pathogenesis of *Aeromonas hydrophila*. *Virulence* 6: 515–522.

Jones N.L., A. Islur, R. Haq, M. Mascarenhas, M.A. Karmali, M.H. Perdue, B.W. Zanke and P.M. Sherman. 2000. *Eschericia coli* Shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family. *Am. J. Physiol. Gastrointest. Liver Physiol.* 278: G811-G819.

Jorgensen J.H. and J.F. Hindler. 2007. New consensus guidelines from the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing of infrequently isolated or fastidious bacteria. *Clin. Infect. Dis*. 44: 280–286.

Joseph S.W. and A. Carnahan. 1994. The isolation, identification, and systematics of the motile *Aeromonas species. Annu. Rev. Fish Dis*. 4: 315–343.

Kali A., R. Kalaivani, P.M.V. Charles and K.S. Seetha. 2016. *Aeromonas hydrophila* meningitis and fulminant sepsis in preterm newborn: A case report and review of literature. *Indian J. Med. Microbiol*. 34: 544–547.

Kampfer P., C. Christmann, J. Swings and G. Huys. 1999. In vitro susceptibilities of *Aeromonas* genomic species to 69 antimicrobial agents. *Syst. Appl. Microbiol*. 22: 662–669.

Kelly K.A., J.M. Koehler and L.R. Ashdown. 1993. Spectrum of extraintestinal disease due to *Aeromonas* species in tropical Queensland, Australia. *Clin. Infect. Dis.* 16: 574–579.

Khan A.A., E. Kim and C.E. Cerniglia. 1998. Molecular cloning, nucleotide sequence, and expression in *Escherichia coli* of a haemolytic toxin (aerolysin) gene from *Aeromonas* trota. *Appl. Environ. Microbiol.* 64: 2473–2478.

Khardori N. and V. Fainstein. 1988. *Aeromonas* and *Plesiomonas* as etiological agents. *Annu. Rev. Microbiol.* 42: 395–419.

Kienzle N., M. Muller and S. Pegg. 2000. *Aeromonas* wound infections in burns. *Burns.* 26: 478–482.

Kirov S.M., T.C. Barnett, C.M. Pepe, M.S. Strom and M.J. Albert. 2000. Investigation of the role of type IV *Aeromonas* pilus (Tap) in the pathogenesis of *Aeromonas* gastrointestinal infection. *Infect. Immun*. 68: 4040–4048.

Ko W.C., H.C. Lee, Y.C. Chuang, C.C. Liu and J.J. Wu. 2000. Clinical features and therapeutic implications of 104 episodes of monomicrobial *Aeromonas* bacteraemia. *J. Infect*. 40: 267–273.

Kumar A., V.N. Bachhil, K.N. Bhilegaonakar and R.K. Agarwal. 2000. Occurrence of enterotoxigenic *Aeromnas* species in foods. *J. Common. Dis*. 32: 169–74.

Lai C.C., L.W. Dingand P.R. Hsueh. 2007. Wound infection and septic shock due to *Aeromonas trota* in a patient with liver disease. *Clin. Infect. Dis*. 44: 1523–1524.

Lai H.T., T.S. Wang and C.C. Chou. 2011. Implication of light sources and microbial activities on degradation of sulfonamides in water and sediment from a marine shrimp pond. *Bior. Tech*. 102: 5017–5023.

Leung K.Y. and R.M.W. Stevenson. 1988. Characteristics and distribution of extracellular proteases from *Aeromonas hydrophila*. *J. Gen. Microbiol.* 134: 151–160.

Libisch B., C.G. Giske, B. Kovacs, T.G. Toth and M. Fuzi. 2008. Identification of the first VIM metallo-β-lactamase-producing multiresistant *Aeromonas hydrophila* strain. *J. Clin. Microbiol*. 46: 1878–1880.

Livermore D.M. 2009. Has the era of untreatable infections arrived? *J. Antimicrob. Chemother.* 64: i29–i36.

Lye D.J. 2011. Gastrointestinal colonization rates for human clinical isolates of *Aeromonas veronii* using a mouse model. *Curr. Microbiol*. 63: 332–336.

Majumdar T., D. Ghosh, S. Datta, C. Sahoo, J. Pal and S. Mazumder. 2007. An attenuated plasmid-cured strain of *Aeromonas hydrophila* elicits protective immunity in *Clarias battrachus L. Fish Shellfish Immunol.* 23: 222–230.

Majumdar T., S. Ghosh, J. Pal and S. Majumder. 2006. Possible role of a plasmid in the pathogenesis of a fish disease caused by *Aeromonas hydrophila*. *Aquaculture* 256: 95–104.

Manna S.K., P. Maurye, C. Dutta and G. Samanta. 2013. Occurrence and virulence characteristics of *Aeromonas* species in meat, milk and fish in india. *J. Food. Saf*. 33: 461–469.

Marcel K.A., A.A. Antoinette and D. Mireille. 2002. Isolation and characterization of *Aeromonas* species from an eutrophic tropical estuary. *Marine Pollut. Bull*. 44: 1341–1444.

Marchandin H., S. Godreuil, H. Darbas, H.J. Pierre, E.J. Bilak, C. Chanal and R. Bonnet. 2003. Extended-spectrum β-lactamase TEM-24 in an *Aeromonas* clinical strain: acquisition from the prevalent *Enterobacter aerogenes* clone in France. *Antimicrob. Agents Chemother.* 47: 3994–3995.

Martin-Carnahan A. and S.W. Joseph. 2005. Genus I. *Aeromonas* Stanier 1943, 213AL, pp. 557–578. In D.J. Brenner, N.R. Krieg, J.T. Staley, and G.M. Garrity (eds). *Bergey's manual of systematic bacteriology*, 2nd ed., vol. 2, Part B. Springer, New York, NY.

Martinez-Murcia A.J., M.J. Saavedra, V.R. Mota, T. Maier, E. Stackebrandt and S. Cousin. 2008 *Aeromonas aquariorum* sp. nov., isolated from aquaria of ornamental fish. *Int. J. Syst. Evol. Microbiol.* 58: 1169–1175.

Martinez-Murcia A., R. Beaz-Hidalgo, A. Navarro, M.J. Carvalho, M. Aravena-Roman, A. Correia, M.J. Figueras and M.J. Saavedra. 2016. *Aeromonas lusitana* sp. nov., isolated from untreated water and vegetables. *Curr. Microbiol.* 72: 795–803.

Minana-Galbis D., M. Farfan, M.C. Fuste and J.G. Loren. 2004. *Aeromonas molluscorum* sp. nov., isolated from bivalve molluscs. *Int. J. Syst. Evol. Microbiol.* 54: 2073–2078.

Minana-Galbis D., M. Farfan, M.C. Fuste and J.G. Loren. 2007. *Aeromonas bivalvium* sp. nov., isolated from bivalve molluscs. *Int. J. Syst. Evol. Microbiol.* 57: 582–587.

Miyake M., K. Iga, C. Izumi, A. Miyagawa, Y. Kobashi and T. Konishi. 2000. Rapidly progressive pneumonia due to *Aeromonas hydrophila* shortly after near-drowning. *Intern. Med*. 39: 1128–1130.

Moawad M.R. and M. Zelderman. 2002. *Aeromonas hydrophila* wound infection in elective surgery. *J. Wound Care.* 11: 210–211.

Mohan B., N. Sethuraman, R. Verma and N. Taneja. 2017. Speciation, clinical profile & antibiotic resistance in *Aeromonas* species isolated from cholera-like illnesses in a tertiary care hospital in north India. *Indian J. Med. Res*. 146: 53–58.

Mohanty B.R., J. Mishra, S. Das, J.K. Jena and P.K. Sahoo. 2008. An outbreak of Aeromoniasis in an organized composite carp culture farm in India: experimental pathogenicity and antibiogram study. *J. Aqua*. 16: 27–37.

Monette S., A.D. Dallaire, M. Mingelbier, D. Groman, C. Uhland, J.P. Richard, T.G. Paillard, L.M. Johannson, D.P. Chivers, H.W. Ferguson, F.A. Leighton and E. Simko. 2006. Massive mortality of common carp (Cyprinus carpio carpio) in the St. Lawrence river in 2001: diagnostic investigation and experimental induction of lymphocytic encephalitis. *Vet. Pathol*. 43: 302–310.

Mosser T., E.T. Reboul, S.M. Colston, J. Graf, M.J. Figueras, E.J. Bilak and B. Lamy. 2015. Exposure to pairs of *Aeromonas* strains enhances virulence in the *Caenorhabditis elegans* infection model. *Front. Microbiol*. 6: 12218.

Moyer N.P. 1987. Clinical significance of *Aeromonas* species isolated from patients with diarrhea. *J. Clin. Microbiol.* 25: 2044–2048.

Nagar V., L.P. Godambe, J.R. Bandekar and R. Shashidhar. 2017. Biofilm formation by *Aeromonas* strains under food-related environmental stress conditions. *J. Food Process Preserv*. 41: 13182.

Namba A., N. Mano, H. Takano, T. Beppu, K. Ueda and H. Hirose. 2008. OmpA is an adhesion factor of *Aeromonas veronii*,an optimistic pathogen that habituates in carp intestinal tract. *J. Appl. Microbiol.* 105: 1441–1451.

Nawaz M., S.A. Khan, A.A. Khan, K. Sung, Q. Tran, K. Kerdahi and R. Steele. 2010. Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. *Food Microbiol.* 27: 327–331.

Nelson K.L., R.A. Brodsky and J.T. Buckley. 1999. Channels formed by subnanomolar concentrations of the toxin aerolysin trigger apoptosis of T lymphomas. *Cell Microbiol*. 1: 69–74.

Nieto T.P., Y. Santos, L.A. Rodriguez and A.E. Ellis. 1991. An extracellular acetylcholinesterase produced by Aeromonas hydrophila is a major lethal toxin for fish. *Microb. Pathog.* 1: 101–110.

Padmaja K., V. Lakshmi and K.V.D. Murthy. 2013. Sepsis due to *Aeromonas hydrophila. Int. J. Infect. Control* 9: 1–4.

Pal A. and S. Bhattacharjee. 2011. Isolation and characterization of aeromonads from North Bengal, India. *NBU J. Anim. Sc.* 5: 47–56.

Pang M., J. Jiang, X. Xie, Y. Wu, Y. Dong, A.H.Y. Kwok, W. Zhang, H. Yao, C. Lu, F.C. Leung and others. 2015. Novel insights into the pathogenicity of epidemic *Aeromonas hydrophila* ST251 clones from comparative genomics. *Sci. Rep*. 5: 9833.

Pidiyar V., A. Kaznowski, N.B. Narayan, M. Patole and Y.S. Shouche. 2002. *Aeromonas culicicola* sp. nov., from the midgut of *Culex quinquefasciatus*. *Int. J. Syst. Evol. Microbiol.* 52: 1723–1728.

Pinna A., L.A. Sechi, S. Zanetti, D. Usai and F. Carta. 2004. *Aeromonas caviae* keratitis associated with contact lens wear. *Ophthalmology* 111: 348–351.

Piotrowska M. and M. Popowska. 2015. Insight into the mobilome of *Aeromonas* strains. *Front. Microbiol.* 6: 1–16.

Ponnusamy D., E.V. Kozlova, J. Sha, T.E. Erova, S.R. Azar, E.C. Fitts, M.L. Kirtley B.L. Tiner, J.A. Andersson, C.J. Grim and others. 2016. Cross-talk among flesh-eating *Aeromonas hydrophila* strains in mixed infection leading to necrotizing fasciitis. *Proc. Natl. Acad. Sci.* 113: 722–727.

Pradhan K. and J. Pal. 1990. Drug sensitivity testing of bacteria isolated from ulcerative diseases of air breathing fishes. *J. Parasitol. App. Anim. Biol*. 2: 15–18.

Pradhan K. and J. Pal. 1993. Drug sensitivity testing of bacteria isolated from ulcerative disease of air-breathing fishes. *J. Parasitol. Appl. Anim. Biol.* 2: 15–18.

Rabaan A.A., I. Gryllos, J.M. Tomas and J.G. Shaw. 2001. Motility and the polar flagellum are required for *Aeromonas caviae* adherence Hep-2 cells. *Infect. Immun.* 69: 4257–4267.

Reshma J.M., R. Amsaveni, M. Sureshkumar, G. Vivekanandhan. 2015. Screening of haemolytic *Aeromonas sp*. Isolated from marine fish samples. *Int. J. Adv. Res.* 3: 1004–1008.

Rhodes G., G. Huys, J. Swings, P. Mcgann, M. Hiney, P. Smith and R.W. Pickup. 2000. Distribution of Oxytetracycline Resistance Plasmids between *Aeromonads* in Hospital and Aquaculture Environments: Implication of Tn*1721* in Dissemination of the Tetracycline Resistance Determinant Tet A. *Appl. Environ. Microbiol.* 66: 3883–3890.

Rosenblatt-Farrell N. 2009. The landscape of antibiotic resistance. *Environ. Health Perspec*. 117: 245–250.

Rouf M.A. and M.M. Rigney. 1993. Bacterial Florae in Larvae of the Lake Fly *Chironomus plumosus. Appl. Environ. Microbiol.* 59: 1236–1241.

Saha D. and J. Pal. 2002. In vitro antibiotic susceptibility of bacteria isolated from EUS-affected fishes in India. *Lett. Appl. Microbiol.* 34: 311–316.

Saha P. and T. Chakrabarti. 2006. *Aeromonas sharmana* sp. nov., isolated from a warm spring. *Int. J. Syst. Evol. Microbiol.* 56: 1905–1909.

Sandvig T. 2001. Shiga toxins. *Toxicon*. 39: 1629–1635.

Saurez G., B.K. Khajanchi, J.C. Sierra, T.E. Erova, J. Sha and A.K. Chopra. 2012. Actin cross linking domain of *Aeromonas hydrophila* repeat in toxin A (RtxA) induces host cell rounding and apoptosis. *Gene* 506: 369–376.

Scheffer J., W. Konig, V. Braun and W. Goebel. 1988. Comparison of four hemolysin producing organisms (*Escherichia coli*, *Serratia marcescens, Aeromonas hydrophila*, and *Listeria monocytogenes*) for release of inflammatory mediators from various cells. *J. Clin. Microbiol*. 26: 544–551.

Schubert R.H.W., M. Hegaz and W. Wahlig. 1990. *Aeromonas enteropelogenes* species nova. *Hyg.Med*. 15: 471–472.

Sha J., E.V. Kozlova and A.K. Chopra. 2002. Role of various enterotoxins in *Aeromonas hydrophila*-induced gastroenteritis: generation of enterotoxin gene-deficient mutants and evaluation of their enterotoxic activity. *Infect. Immun*. 70: 1924–1935.

Sha J., T.E. Erova, R.A. Alyea, S. Wang, J.P. Olano, V. Pancholi and A.K. Chopra. 2009. Surface-Expressed Enolase Contributes to the Pathogenesis of Clinical Isolate SSU of *Aeromonas hydrophila. J. Bacteriol.* 191: 3095–3107.

Sierra J.C., G. Suarez, J. Sha, W.B. Baze, S.M. Foltz and A.K. Chopra. 2010. Unraveling the mechanism of action of a new type III secretion system effector AexU from *Aeromonas hydrophila. Microb. Pathog.* 49: 122–134.

Sinha S., S. Chattopadhyay, S.K. Bhattacharya, G.B. Nair and T. Ramamurthy. 2004. An unusually high level of quinolone resistance associated with type II topoisomerase mutations in quinolone resistance-determining regions of *Aeromonas caviae* isolated from diarrhoeal patients. *Res. Microbiol*. 155: 827–829.

Tang H.J., C.C. Lai, H.L. Lin and C.M. Chao. 2014. Clinical Manifestations of Bacteremia caused by *Aeromonas* Species in Southern Taiwan. *Plos One*. 9: 91642.

Tena D., C. Aspiroz, M.J. Figueras, A. Gonzalez-Praetorius, M.J. Aldea, A. Alperi and J. Bisquert. 2009. Surgical site infection

due to *Aeromonas species*: report of nine cases and literature review. *Scand. J. Infect. Dis*. 41: 164–170.

Tewari R., M. Dudeja, S. Nandy and A.K. Das. 2014. Isolation of *Aeromonas salmonicida* from human blood sample: a case report. *J. Clin. Diag. Res*. 8: 139–140.

Toranzo A.E., J.L. Barja, R.R. Colwell and F.M. Hetrick. 1983. Characterization of Plasmids in Bacterial Fish Pathogens. *Infect. Immun*. 39: 184–192.

Tsai M.S., C.Y. Kuo, M.C. Wang, H.C. Wu, C.C. Chien and J.W. Liu. 2006. Clinical features and risk factors for mortality in *Aeromonas* bacteremic adults with hematologic malignancies. *J. Microbiol. Immunol. Infect*. 39: 150–154.

Umelo E. and T.J. Trust. 1997. Identification and molecular characterization of two tandemly located flagellin genes from *Aeromonas salmonicida* A449. *J. Bacteriol.* 179: 5292–5299.

Walsh T.R., J. Weeks, D.M. Livermore and M.A. Toleman. 2011. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet. Infect. Dis.* 11: 355–362.

Walsh T.R., R.A. Stunt, J.A. Nabi, A.P. MacGowan and P.M. Bennett. 1997. Distribution and expression of β-lactamase genes among *Aeromonas spp*. *J. Antimicrob. Chemother*. 40: 171–178.

Wang G., K.D. Tyler, C.K. Munro and W.M. Johnson. 1996. Characterization of cytotoxic, haemolytic *Aeromonas caviae* clinical isolates and their identification by determining presence of a unique hemolysin gene. *J. Clin. Microbiol.* 34: 3203–3205.

Wang G., C.G. Clark, C. Liu, C. Pucknell, C.K. Munro, T.M.A.C. Kruk, R. Caldeira, D.L. Woodward and F.G. Rodgers. 2003. Detection and characterization of the Hemolysin Genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. *J. Clin. Microbiol.* 41: 1048–1054.

Yamamoto T., T. Ishii, M. Sanaka, M. Saitoh and Y. Kuyama. 2004. Ileal ulcers due to *Aeromonas hydrophila* infection. *J. Clin. Gastroenterol*. 38: 911.

Yu H.B., Y.L. Zhang, Y.L. Lau, F. Yao, S. Vilches, S. Merino, J.M. Tomas, S.P. Howard and K.Y. Leung. 2005. Identification and Characterization of Putative Virulence Genes and Gene Clusters in *Aeromonas hydrophila* PPD134/91. *Appl. Environ. Microbiol.* 71: 4469–4477.

Zhiyong Z., L. Xiaoju and G. Yanyu. 2002. *Aeromonas hydrophila* infection: clinical aspects and therapeutic options. *Rev. Med. Microbiol*. 13: 151–162.

Zhou L.J., G.G. Ying, J.L. Zhao, J.F. Yang, L. Wang, B. Yang and S. Liu. 2011. Trends in the occurrence of human and veterinary antibiotics in the sediments of the Yellow River, Hai River and Liao River in northern China. *Environ. Pollut*. 159: 1877–1885.

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MINIREVIEW

Brucella – Virulence Factors, Pathogenesis and Treatment

PATRYCJA GŁOWACKA[1]*, DOROTA ŻAKOWSKA[1], KATARZYNA NAYLOR[2], MARCIN NIEMCEWICZ[1] and AGATA BIELAWSKA-DRÓZD[1]

[1] Biological Threats Identification and Countermeasure Center of the General Karol Kaczkowski Military Institute of Hygiene and Epidemiology, Puławy, Poland
[2] Lublin Medical University, Department of Didactics and Medical Simulation, Lublin, Poland

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Abstract

Brucellae are Gram-negative, small rods infecting mammals and capable of causing disease called brucellosis. The infection results in abortion and sterility in domestic animals (sheeps, pigs, rams etc). Especially dangerous for humans are: *Brucella melitensis*, *Brucella suis*, *Brucella abortus*, and *Brucella canis* that trigger unspecific symptoms (flu-like manifestation). *Brucella* rods are introduced via host cells, by inhalation, skin abrasions, ingestion or mucosal membranes. The most important feature of *Brucella* is the ability to survive and multiply within both phagocytic and non-phagocytic cells. *Brucella* does not produce classical virulence factors: exotoxin, cytolisins, exoenzymes, plasmids, fimbria, and drug resistant forms. Major virulence factors are: lipopolysaccharide (LPS), T4SS secretion system and BvrR/BvrS system, which allow interaction with host cell surface, formation of an early, late BCV (*Brucella* Containing Vacuole) and interaction with endoplasmic reticulum (ER) when the bacteria multiply. The treatment of brucellosis is based on two-drug therapy, the most common combinations of antibiotics are: doxycycline with rifampicin or fluoroquinolones with rifampicin. Currently, also other methods are used to disrupt *Brucella* intracellular replication (tauroursodeoxycholic acid or ginseng saponin fraction A).

Key words: *Brucella*, endoplasmic reticulum, macrophage, replication, virulence factors

Introduction

Brucella is a genus of bacteria belonging to the phylum Proteobacteria, class Alphaproteobacteria, order Rhizobiales, family Brucellaceae. Alphaproteobacteria is a very diverse group as to this class belongs both, the pathogens associated with plants: *Agrobacterium* spp., *Sinorhizobium* spp., *Mesorhizobium* spp. and the pathogens, which cause dangerous infections of animals, *e.g. Ricketsia* spp., *Bartonella* spp., *Brucella* spp. and many others (Dwight and Bowman, 2011).

Brucella genus is responsible for brucellosis, a severe febrile disease. Brucellosis is a worldwide problem, causing abortion and infertility in domestic and wild animals (Lapaque *et al.*, 2005). Infection factors are aerobic, small, Gram-negative rods. *Brucella*, a genus discovered in 1887 by David Bruce, contains the following species: *Brucella suis*, *Brucella ovis*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella neotomae, Brucella ceti*, *Brucella pinnipedialis*, *Brucella microti, Brucella inopinata*, *Brucella papionis*, *Brucella vulpis* and other strains without standing in nomenclature, that include environmental samples (Galińska and Zagórski, 2013; Whatmore *et al.*, 2014; Scholz *et al.*, 2016). Some species contain biovars, for example: *B. suis* have five biovars, *B. melitensis* contain three and *B. abortus* – nine biovars (Mizak *et al.*, 2014). Most of these species infect mainly specific hosts. *B. abortus* causes disease in cattle and infections usually lead to abortion; whereas *B. suis* is responsible for brucellosis in pigs, resulting in reproductive problems. Sheep are hosts for *B. melitensis*; infection causes impaired fertility. *B. ovis* is an etiological factor in sterility of rams (Megid *et al.*, 2010). Currently, about 500 000 cases of human brucellosis have been reported worldwide annually (Byndloss and Tsolis, 2016). Brucellosis is an endemic zoonosis with infection predominantly occurring in Middle East, Mediterranean rim (Portugal, Spain, Greece), Asia, Africa, South and Central America where the intake of dairy products is high, and protection of animal health is insufficient (Rubach *et al.*, 2013). There are single cases reported in Poland, however connected with occupational exposure or with traveling to Mediterranean countries (Galińska and Zagórski, 2013). *B. abortus* and *B. suis* are isolated not only from livestock but also from different wildlife species (bears, buffalo, bison, caribu, camelids, elk,

* Corresponding author: P. Głowacka, Biological Threats Identification and Countermeasure Center of the General Karol Kaczkowski Military Institute of Hygiene and Epidemiology, Puławy, Poland; **e-mail: ppaczka@wihe.pulawy.pl**

ferrets, deer, foxes, rodents, rabbits, wolves) and marine mammals (dolphins, dugongs, manatees, otters, sea porpoise) (Coelho *et al*., 2015). *B. melitensis* is rarely encountered in wildlife, nonetheless individual cases have been reported in ibex and in chamois in Alps. *B. ovis* and *B. canis* have not been detected in wildlife in Europe up to date. *B. pinnipedialis* and *B. ceti* cause the most common infections in marine mammals. Birds are resistant to *Brucella* infection, whereas fish seem to be susceptible to *B. pinnipedialis* and *B. ceti* infections (Godfroid *et al.,* 2013). Infection is transmitted through close contact and during a common pasture.

Brucellosis is transferred from animals to humans, frequently human to human transmission occurs (Osman *et al.,* 2016). Especially dangerous for humans are: *B. melitensis*, *B. suis*, and *B. abortus*, *B. canis.* Brucellosis in human presents with symptoms like influenza: undulating fever, depression, weight loss, hepatomegaly, and splenomegaly (Bingöl *et al.*, 1999). Mainly human cases are connected to occupational risk or consumption of unprocessed dairy products (Boschiroli *et al.*, 2001; de Figureido *et al.*, 2015). *Brucella* rods can enter via host cells by inhalation, ingestion, skin abrasion, or mucosal membranes (Franco *et al.*, 2007). After penetration into host, rods multiply in lymph nodes; afterward, they penetrate other organs (Galińska and Zagórski, 2013). *Brucella,* can modify immune response in host cells; it has an affinity to the cells of specific tissues, *e.g.* placental trophoblast in fetal lung, pregnant females or reproductive system (de Figureido *et al.*, 2015). Brucellosis causes enlargement of lymph nodes, liver and spleen (Perkins *et al.*, 2010). Pathogenicity of *Brucella* is dependent on their ability to multiply and survive within macrophages (Sangari and Agűero, 1996; Christopher *et al.*, 2010).

Characteristics of *Brucella*

Species of the genus *Brucella* belong to small coccobacilli, measuring about 0,6–1,5 μm (Alton and Forsyth, 1996). They occur in single forms; rarely they create pairs or chains (Mizak *et al.*, 2014). *Brucella* are nonspore forming and non-motile Gram-negative cocobacilli (GNCB) (Alton and Forsyth, 1996). *Brucella* is an intracellular pathogen, during an infection it survives and multiplies in macrophages; the bacteria adapt to the acidic pH, low levels of oxygen, and low levels of nutrients (Kőhler *et al.*, 2002).

Lipopolisaccharide (LPS) is an essential element of structure building in each Gram-negative bacterial cell. *Brucella* is a genus that creates two forms of LPS. The smooth forms present complete LPS in the outer membrane, the rooth phenotype does not contain polisaccharide O-chain (Lapaque *et al.*, 2005; Seleem *et al.*, 2008). These infectious agents are able to produce cytochrome oxidase, catalase, and most of them are able to hydrolyze urea (Iowa State University, 2009). *Brucella* does not produce classical pathogenic factors, such as: exotoxin, cytolisins, exoensymes, exoproteins, capsules, plasmids, fimbria, and drug resistant forms (Seleem *et al.*, 2008; Baldi and Giambartolomei, 2013; Tan *et al.*, 2015). Bacterial cells are able to survive for a prolonged time in water, aborted fetus, soil, dairy products, meat, dung, and dust (Gwida *et al.*, 2010). For isolation of *Brucella* spp. the enrichment and selective media such as Thayer-Martin's medium or Farrell's medium are commonly used. The colonies mature after four to six days of incubation at temperature of 37°C. They can also grow at 28°C, but poorly and slowly. Moreover, these bacteria can grow in both aerobic atmosphere and in 10% CO_2; while, their growth is enhanced without additional CO_2 on a serum dextrose agar (Iowa State University, 2009; Whatmore *et al.*, 2014; Gupte and Kaur, 2015).

A wide range of bacterial detection methods is available. The predominatingly used culture media are: Bacto Tryptose (Difco), Triptcase soy (BBL), Tryptone soya (Oxoid), Triptic soy (Gibco). For culture of blood or body fluid a biphase medium called Castaneda should be used. Castaneda consists of two phases: liquid and solid closed in bottle. Liquid medium contains 1–2% of sodium citrate. Sample (5–10 ml) is added to the medium and incubated in 37°C in perpendiculary standing bottle in 10% carbon dioxide atmosphere (Gupte and Kaur, 2015). Serological tests are used to detect infection by examination of a specific antibodies level in serum. In the first week of *Brucella* infection the titres of IgM are dominant, but in the second week IgG class is prevalent. After four weeks, both types of antibodies reach a peak; durable, high titres of IgG can evidence failure in treatment (Al Dahouk *et al.*, 2013). Serum Agglutination Test (SAT) and Enzyme linked immunosorbent assay (ELISA) are the most common serological tests used for diagnosis of brucellosis. SAT is based on a survey of agglutination titer of different serum dilution against *Brucella* cell suspension (Alshaalan *et al.*, 2014). ELISA depends on detection of antibodies against the antigen – smooth LPS in serum (Gerasu and Kassa, 2016). The most effective methods for detection of brucellosis are molecular techniques (classical PCR, real-time PCR). The PCR method applies various pairs of primers to amplify different fragments of the genome. The examples of genes used to identification of *Brucella* spp. are: BCSP 31 (primers: B4/B5), sequence of 16S rRNA (primers: F4/R2), omp2 gene (primers: JPF/JPR) (Baddour and Alkhalifa, 2008).

Virulence factors

Lipopolisaccharide. LPS is an essential virulence factor of *Brucella*. LPS consists of lipid A, oligosaccharide core and O-antigen in Gram-negative bacteria.

Lipopolisaccharide is different and non-classical in *Brucella* as compared to other Gram-negative bacteria, for example *E. coli* (Cardosos *et al.*, 2006; Christopher *et al.*, 2010). Lipopolisaccharide from *Brucella* strains is less toxic and less active than the classical LPS isolated from *E. coli*. Classical LPS causes a high pyrogenicity, while non-classical LPS shows low pyrogenicity, being a weak inducer of tumor necrosis factor (Christopher *et al.*, 2010). Three features distinguishing it from other Gram-negatives characterize lipid A found in *B. abortus*: i) the fundamental component is diaminoglucose instead glucosamine, ii) longer acyl groups, and iii) lipid A is connected to the core by amide bonds, instead ester and amide bounds (Lapaque *et al.*, 2005). In strains with smooth colonies, the smooth LPS, (S-LPS) contains: i) lipid A, that consists of two types of aminoglycose, and fatty acid besides β-hydroxymiristic acid, ii) core comprises mannose, glucose, quinovosamine, and iii) O-chains are composed of 4-formamido-4,6-dideoxymannose. The structure of the R-LPS in strains with rough colonies is similar to the S-LPS, except for O-chains, which are reduced or absent (Corbel, 1997). *B. suis* has S-LPS. The O-chain connects with lipid rafts on the macrophage surface and the bacteria enter the cell. *Brucella* strains with R-LPS, for example *B. ovis* or *B. canis* do not connect with lipid rafts and rapidly connect with lysosomes (Lapaque *et al.*, 2005). The strains with S-LPS are able to restrain host cell apoptosis by the interaction of the O-chain with TNF-α (tumor necrosis factor). Thus, dead cells do not release specific factors, therefore they do not activate the immune system and *Brucellae* are able to avoid host immune surveillance (Fernandez--Prada *et al.*, 2003).

Type IV secretion system (T4SS). T4SS is a multiprotein complex and participates in secretion of bacterial macromolecues (Cascales and Christie, 2003). This system is typified by v*irB* operon encoding 12 proteins (11 860 bp) and exhibits in *Brucella* spp. with a high degree of similarity to T4SSs found in rhizobia, for example in phytopatogenic *Agrobacterium tumefaciens* (O'Callaghan *et al.*, 1999). Expression of the *virB* operon is regulated by the regulator of quorum-sensing – VjbR (Seleem *et al.*, 2008). The wild strains of *Brucella* are able to multiply only in the endoplasmic reticulum. *VirB* mutants of *Brucella* spp. are unable to multiply within the endoplasmic reticulum, it can result from the incapability to reach the ER, or multiply within (Delure *et al.*, 2001). In macrophages, rods of *Brucella* spp. are localized in *Brucella*-containing vacuole (BCV); this organelle interacts with the ER and is responsible for formation of specialized brucellae-multiplication compartment (Kőhler *et al.*, 2002). The acquisition of endoplasmic reticulum membrane depends on a functional *virB* secretion system – T4SS (Celli *et al.*, 2003).

Superoxide dismutase and catalase. Macrophages with *Brucella* produce reactive oxygen intermediates (ROIs), this is a primary mechanism of destruction of the bacteria ingested, and it also prevents their intracellular replication (Gee *et al.*, 2005; Seleem *et al.*, 2008). The following ROIs: O^{2-} (superoxide), H_2O_2 (hydrogen peroxide), OH^- (hydroxyl radical) are very detrimental for cell structure. The production of enzymes is the main line of defense, counteracting reactive oxygen intermediates. These enzymes include superoxide dismutase (SOD) and catalase (Gee *et al.*, 2005). SOD (metalloenzyme) is encoded by *sod* sequence. An enzyme contains iron, magnesium, or zinc and copper at its active site (Benov and Fridovich, 1994). SOD is responsible for dismutation of O^{2-} (superoxide) to H_2O_2 (hydrogen peroxide) and O_2 (oxygen) – transfer from one molecule to another ($2O^{2-}+2H^+ \rightarrow H_2O_2 + O_2$) (Gopal and Elumalai, 2017). Some species possess two types of SOD (*B. abortus*, *B. melitensis*, *B. suis*). The first is cytoplasmic – a Mn cofactor – SodA. SodA neutralizes endogenously generated O^{2-} – product of aerobic metabolism. The second one, SodC is periplasmic Cu, Zn-SOD, an enzyme responsible for neutralizing exogenously generated O^{2-} and protection from the respiratory burst within macrophages (Beck *et al.*, 1990, Seleem *et al.,* 2008, Martin *et al.,* 2012).

Catalase decomposes hydrogen peroxide into oxygen and water. Catalase activity is limited to the periplasmic space, where together with Cu-Zn SOD leave external sources of ROI unchanged (Kim *et al.*, 2000). Catalase is not necessary virulence factor, the other enzymes can compensate lack of this enzyme in catalase mutants, *e.g.* alkyl hydroperoxide reductase or enzymes involved in DNA repair mechanisms (Seleem *et al.*, 2008). Catalase is encoded by a sequence similar to *katE* gene of *Escherichia coli*. *B. abortus*, *B. melitensis* and *B. suis* catalase production is regulated by an increased external level of H_2O_2 (Gee *et al.,* 2004).

Cyclic β-1-2-glucans (CβG). *Brucella* CβG belongs to II OPGs (Osmoregulated periplasmic glucans) family (Bohin, 2000). *B. abortus* CβG impacts intracellular trafficking by acting on lipid rafts on macrophage surface. These glucans participate in control of the phagosome-lysosome fusion. Mutants are destroyed in phagolysosome and they are not able to multiply. Even more, mutants treated by CβG are able to control vacuole maturation and lysosome fusion, so they can reach to the ER and replicate there (Arellano-Reynoso *et al.*, 2005).

Urease. In *Brucella* there are non-identical urease operons in two separate genomes. Urease is a metalloenzyme, that decomposes urea to carbonic acid and to ammonium form, and it results in pH increase. This feature enables its survival in acid environment (Seleem *et al.*, 2008). In I chromosome, there are two

urea-operons: *ure-1* and *ure-2*, separated by 1 Mb of DNA. *Ure-1* and *ure-2* encode structural genes: *ureA*, *ureB*, *ureC* and accessory genes: *ureD*, *ureE*, *ureF*, *ureG* (Mobley *et al.*, 1995). That urease may protect *Brucella* during passage through the digestive tract (stomach), when the bacteria access their host through the oral route (Bandara *et al.*, 2007). Urease is produced by all bacteria belonging to the genus *Brucella* but *B. ovis* (Sangari *et al.*, 2007).

Cytochrome oxidase. Cytochrome oxidase is an enzyme facilitating *Brucella*'s survival inside the macrophages, where oxygen availability is limited. There are two operons in genome encoding two types of high oxygen-affinity oxidases: cytochrome cbb3-type and cytochrome bd (ubiquinol oxidases) oxidases. Cytochrome cbb3 oxidase is expressed *in vitro* and allows for colonization of anoxic tissues (maximal action in microaerobiosis). Cytochrome bd oxidase is expressed during intracellular multiplication and enables adjustment to the replicative niche (Loiser-Meyer *et al.*, 2005), by restraining the creation of oxidative free radicals and detoxification of compartment inside the cell (Endley *et al.*, 2001).

Alkyl hydroperoxide reductase (AhpC, AhpD). These enzymes attempt protection against oxygen radical and reactive nitrogen (Chen *et al.*, 1998). *AhpC* and *ahpD* are organized in an operon under one promoter control. *AhpC* mutants are more sensitive to peroxide killing and are vulnerable to spontaneous mutagenesis (DelVecchio *et al.*, 2002; Seleem *et al.*, 2008).

Nitric oxide reductase (NorD). Reduction of nitrate to dinitrogen gas is an essential process for bacteria in case of oxygen deficiency inside the cell; this process allows for respiration of nitrate (Stevanin *et al.*, 2005). The infected macrophages produce nitric oxide (NO), and *Brucella* can use it for own purposes. *Brucella* NorD consists of four types of reductases: Nir – nitrite reductase, Nar – nitrate reductase, Nor – nitric oxide reductase and Nos – nitrous oxide reductase, called the nitrification island. Possibility concerning productions of this enzymes helps to protect *Brucella* against low-oxygen conditions inside macrophages (Seleem *et al.*, 2008).

***Brucella* virulence factor A (BvfA).** Periplasmic protein that occurs only in genus *Brucella*; there are no homologous sequences in Gen Bank. The *bvfA* expression is induced in macrophages, through phagosome acidification. Presumably this protein is involved in forming the replication intracellular niche. BvfA function is not precisely identified (Lavigne *et al.*, 2005).

Base excision repair (BER). *XthA* gene encodes exonuclease III, which takes part in the base excision repair of DNA. Two different sequences of *xthA* occur in the *Brucella* genome: *xthA-1* and *xthA-2*. *XthA-1* mutants exhibit increased sensitivity to reactive oxygen species (ROS), so this enzyme is responsible for protection against oxidative destruction (Seleem *et al.*, 2008).

BvrR/BvrS system. The analysis of *Brucella* genomic library has confirmed an occurrence of two open reading frames: *bvrR* and *bvrS*. The *bvrR* encodes BvrR proteins (237 amino acid) and *bvrS* encodes BvrS (601 amino acid). There are two potential promoters (–10 and 35 seq. located 50 bp upstream ORF of *bvrR*), and ribosome-binding sequence (9 bp upstream of the first codon) (Sola-Landa *et al.*, 1998). BvrR exhibits resemblance to response regulators proteins, as N-terminal domain is composed of highly conserved aminoacids: aspartic (pos: 14, 15, 58) and lysine (pos: 107). C-terminal domain showed high similarity sequence to OmpR family; therefore, this protein can be included as part of this family (Mizuno and Tanaka, 1997; Martínez-Nūñez *et al.*, 2010). The protein is composed of three highly conserved domains: N-terminal sensing, periplasmic domain together with transmembrane component, cytoplasmic domain with distinctive histidine residue and C-terminal ATP-binding domain (Viadas *et al.*, 2010). BvrS includes four highly conserved regions on C-terminal domain: H, N, D/F, and G. This feature causes BvrS homologous to sensor proteins of the histidine protein kinase family (Stock *et al.*, 1995). BvrS is located in the cell membrane (Martínez-Nūñez *et al.*, 2010). Brucella BvrR/BvrS are the best characterized components of the virulence system; mutants are incapable of invasion, prevention phagosome-lysosome fusion and intracellular replication. BvrR/BvrS system is a regulator of expression of multiple genes (Viadas *et al.*, 2010). These proteins affect the transcription of the membrane proteins: Omp3b (Omp22) or Omp3a (Omp25a) and have the influence on other non-protein membrane molecules and hence on functional and structural membrane homeostasis (Manterola *et al.*, 2007). *BvrR*/*bvrS* mutants show structural changes in LPS, but O-chains seem to be undisturbed. These mutants are incapable of activation of GTPase (Cdc42) before entry into the cell, so they persist extracellularly and in consequence they do not infect the cell (Guzmán-Verri *et al.*, 2001). BvrR/BvrS is also responsible for limited lysosome fusion and intracellular trafficking (López-Goñi *et al.*, 2002).

Brucella BvrR/BvrS regulatory system action activate sensor domain of the BvrS protein by environmental signals through kinase activity. Additionally, BvrS causes phosphorylation and activation of BvrR protein. BvrR activates transcription of *omp3a*, *omp3b* and other genes responsible for lipid A structure and perhaps core of LPS. In consequence, *bvrS*/*bvrR* mutants are more sensitive to cationic peptides and display increased permeability for surfactants (López-Goñi *et al.*, 2002; Seleem *et al.*, 2008). The influence of Omp3a and Omp3b on virulence remains unexplained in details (Manterola *et al.*, 2007).

It has been proven that BvrR/BvrS two-component system regulate the expression of *virB* by positive stimulation of *vjbR* transcription. *VjbR* transcriptional factor interacts with *virB* promoter (Martínez-Nūñez *et al.*, 2002).

Role of virulence factors in chronic persistence. Evading an immunological response to *Brucella* antigens depends on LPS structure. The appearance of elongated fatty acid on the lipid A (*Brucella*-C_{28} compared to others Enterobacteriaceae $C_{12} - C_{16}$) leads to poor activation of TLR4 (Tool-like receptor 4) (de Figueiredo *et al.*, 2015; Byndloss and Tsolis, 2016). The other feature of *Brucella* spp. LPS is core oligosaccharide glycosylation pattern that prevents a connection of the bacteria with TLR4 (co-receptor MD-2) (Byndloss and Tsolis, 2016). Toll-like receptors, the transmembrane proteins, act as PRRs (Pattern recognition receptors) and initiate the innate immune responses. TLRs are responsible for the recognition of components of microorganism (Uemattsu *et al.*, 2008). The TLR protein is composed of two domains: extracellular domain that is rich in leucine repeats and it is responsible for recognition of microbial components, and cytoplasmic domain – TIR, involved in signal transmission, activation of intermediate proteins and finally an activation of NF-κβ and cytokines (Radhakrishnan *et al.*, 2009). TLR5 detects flagellin, but flagellin in *Brucella* spp. can avoid interaction with TLR5 as it lacks the domain recognized by TLR. (Andersen-Nissen *et al.*, 2005; Kim, 2015). *Brucella* encoded TcpB/BtpA protein that acts as a following virulence factor (TcpB – *B. melitensis*, Btp1/BtpA – *B. abortus*). Three pathogenic microorganism can produce similar proteins: *Salmonella* spp., *E. coli* and *Brucella* (Radhakrishnan *et al.*, 2009). These proteins contain TIR domain and show similarity to TIRAP (MAL) – a TLR adaptor protein. TcpB promotes a degradation of TIRAP and disrupts TLR4 signalling that result in inhibition of proinflammatory cytokines production and dendritic cell maturation (Newman *et al.*, 2006; Oliveira *et al.*, 2008; Radhakrishnan *et al.*, 2009; Sengupta *et al.*, 2010; Byndloss and Tsolis, 2016). TIRAP triggers recruitment of MyD88 and hence mediates TLR4 and TLR2 signalling (Kagan and Medzhitov, 2006). It seems to be likely that TcpB is able to interact with Death Domain of MyD88 and affect the signalling pathway (Chaudhary *et al.*, 2012). Another group of researchers has proven that *Brucella* encodes another TIR domain-containing protein, called BtpB (present in all *Brucella* strains). This protein reacts with MyD88, inhibits TLR signalling, and disrupts activation of dendritic cells BtpB, restraining TLR2, TLR4, TLR5 and TLR9 signalling; and together with BtpA affects DC maturation and inflects host inflammatory responses (Salcedo *et al.*, 2013).

B. abortus has proline racemase and thus is able to produce anti-inflammatory cytokine IL-10. This cytokine modulates macrophage activity during early phase of infection and leads to persistence and long-term survival of microorganism inside host cells (Byndloss and Tsolis, 2016).

Role of virulence factors in reproductive disease. The investigations performed on bovine placental explants have proven that in early infection with *B. aborus* the suppression of proinflammatory cytokines occured. This process is dependent on BtpB and T4SS proteins (use of mutants of the *virB* and *btpB* genes results in the reverse effect by enhancement of the proinflammatory cytokines production) (Mol *et al.,* 2014). In later phases, 12 h after infection with *B. abortus*, stimulation of proinflammatory cytokines and CXC chemokines production – CXCL6 (GCP-2) and CXCL8 (interleukin 8) takes place. CXCL6 and IL-8 known as neutrophil chemoattractants cause neutrophil influx and have been reported to cause necrotizing placentis after infecting a pregnant cow (Carvalho Neta *et al.*, 2008).

Pathogenesis

Invasion to cell. The *Brucella* strains survive and multiply within both phagocytic and non-phagocytic cells. The main targets for this bacterium are macrophages, dendritic cells and trophoblast cells. However, *Brucella* can also multiply within other cells, for example epithelioid cell (HeLa) or murine fibroblast (NIH3T3) (Pizarro-Cerdá *et al.,* 2000; Celli, 2006; Xavier *et al.*, 2010). *Brucella* translocates across the mucosal epithelial cells layer, where the professional phagocytes (macrophages and DC cells) engulf the bacteria. *Brucella* survives within non-phagocytic cells up to 72 hours after infection, overcomes the epithelial barrier and then penetrates the phagocytic cells. Approximately 10% of these bacteria survive this initial phase. In macrophages the pathogen avoids the host immune response; therefore, it can multiply and spread to other tissues using cellular tropism. The *Brucella* strains penetrate the host cells through a zipper-like mechanism (Gorvel and Moreno, 2002; de Figureido *et al.*, 2015). Bacteria can spread in a host through the lymph nodes and then translocate to the preferred tissues in reproductive tract (Kim, 2015). There, *Brucella* induces acute or chronic infection of reproductive tract that leads to abortion or severe reproductive tract diseases. (He, 2012).

The non-opsonized *Brucella* organisms are internalized through lectin or fibronectin receptors but opsonized by complement and Fc receptors. The opsonized bacteria are more prone to be destroyed within macrophages than non-opsonized ones. The

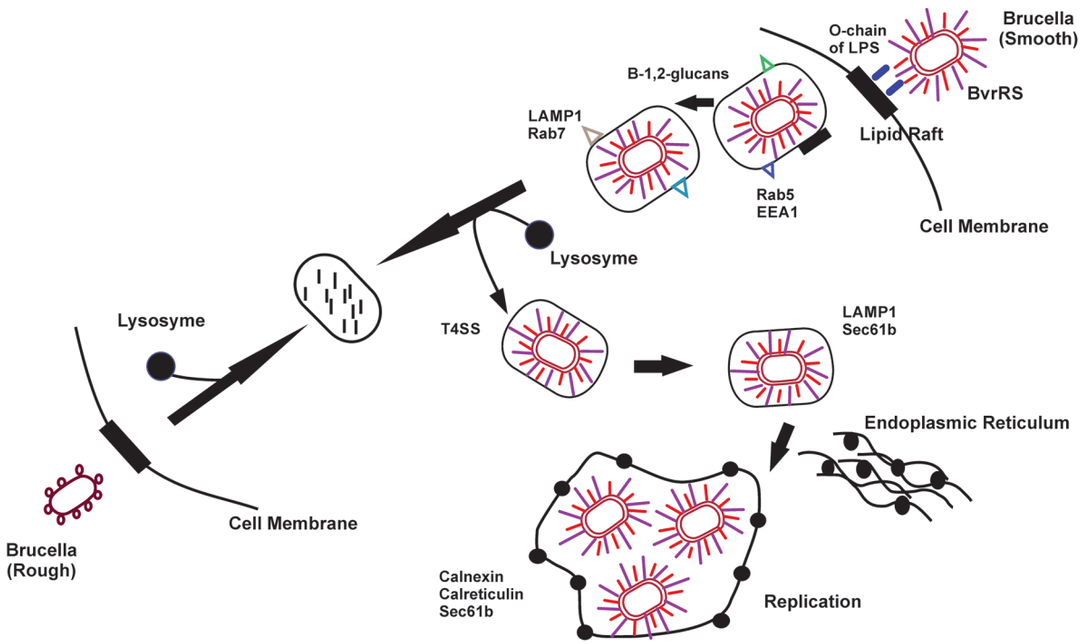

Fig. 1. Mammalian cell invasion and intracellular trafficking.

Smooth *Brucella* invasion into a cell by lipid rafts and acquisition of Rab5 and EEA1 markers – early BCV. β-1,2-glucans present in mature BCV and modification of lipid rafts. Then, the transient BCV interacts with lysosomes, T4SS is activated and it regulates intracellular trafficking from autophagosome to endoplasmatic reticulum (BCV acquires LAMP1 and Sec61β markers – late BCV – occurs only in epithelial cells). BCV acquires the endoplasmic reticulum markers (calnexin, calreticulin and Sec61-β) and the *Brucella* replicates. Rough *Brucella* organisms do not penetrate cell by lipid rafts, and therefore is exterminated.

pathogen binds to receptors containing of sulfated residues and sialic acid on surfaces of epithelial cells (de Figureido *et al.*, 2015).

Penetration into the epithelial cell requires actin polymerization (Kim, 2015). The adhesion of *B. abortus* to the cell surface leads to activation of GTPases of Rho subfamily, *e.g.* Rho, Rac, and Cdc42 (Guzmán-Verri *et al.*, 2001). These proteins are involved in cytoskeletal regulation and have an impact on parasitic bacterial internalization. Cdc42 is the only GTPase activated directly by *B. abortus* during the contact with a non-phagocytic cell. It seems that other GTP-ases (Rho or Rac) are activated indirectly, because their inhibition impede invasion into host cells (Waterman-Storer *et al.*, 1999). Other protein acting as second messengers, *i.e.* cGMP, PIP3-kinase, MAP-kinase and tyrosine kinase are involved in adhesion of bacteria to the host cell surface (Guzmán-Verri *et al.*, 2001).

Adhesion to macrophage surface is also associated with small GTPases activation (Guzmán-Verri *et al.*, 2001) and F-actin polymerization (transient and rapid F-actin accumulation). In the early stages of adhesion Annexin I is also involved, a protein that is implicated in membrane fusion (Kusumawati *et al.*, 2000). Bacterial internalization occurs also by the lipid rafts – microdomains that occur in the macrophage cell membrane. These structure contribute to intracellular trafficking of *Brucella* (Fig.1) (Xavier *et al.*, 2010). Non-opsonized *Brucella* strains internalize in human monocytes and murine macrophages by lipid rafts. This process requires activation of TLR4 and PI3-kinase. However, this process in human dendritic cells is only partly dependent on lipid rafts (von Bargen *et al.*, 2012). *Brucella* strains with lack of O-polysaccharides in LPS (R-LPS) do not penetrate eukaryotic cells by lipid rafts, and thus are exterminated by macrophages (Porte *et al.*, 2003; Gomez *et al.*, 2013). Lipid rafts are rich in cholesterol, GPI (glycosylphosphatidylinositol) and GM1 (gangliosides) (Brown and London, 1998). Lipid rafts-associated proteins: GPI and GM1 as well as cholesterol inosculate with *Brucella*-contain macropinosomes and facilitate internalization with macrophage (Naroeni and Porte, 2002).

Brucella can be recognized by TLRs, but owing to modifications its interaction with TLRs is 10-fold lesser than for Enterobacteria. Hence, the activation of NF-κβ and production of inflammatory cytokines is weaker (de Figureido *et al.*, 2015).

Intracellular trafficking. It has been supposed that intracellular trafficking is not essentially different in professional and non-professional phagocytes (Gorvel and Moreno, 2002). After several minutes of invasion, the bacteria interact with early endosomal network-related compartment – early BCV – *Bru-*

cella Containing Vacuole. This compartment is characterized by Rab5 (GTP-binding protein) and EEA1 (early endosomal antigen 1) markers (Pizarro-Cerdá *et al.*, 1998; Pizarro-Cerdá *et al.*, 2000). β-1,2-glucans are necessary for the regulation of BCV maturation in macrophages as well as in epithelial cells. Additional function of β-1,2-glucan is the modification of rich in cholesterol lipid rafts, which are located on BCV membrane surface (Arellano-Reynoso *et al.*, 2005). The interaction with early endocytic network last about 10 minutes (Pizarro-Cerdá *et al.*, 1998). At this stage, acidification of BCV takes place leading to changes on the bacterial genes expression and enabling intracellular survival (Carvalho Neta *et al.*, 2010). BCV does not interact with late endosome and it avoids fusion with lysosomes (by β-glucans and LPS occurrence) (Gorvel and Moreno, 2002; Celli, 2006). However, early BCV is transformed to intermediate BCV that is LAMP1 and Rab-7 marked (late endosomal/lysosomal markers), indicating that interactions with late endosomal compartments and lysosomes become necessary. What is more, in this step BCV acquires also Rab-interacting lysosomal protein (RILP) that is Rab-7 effector (Starr *et al.*, 2008; von Bargen *et al.*, 2012; Gomez *et al.*, 2013). The interaction between BCV and late endosomes/lysosomes is transitional and controlled. This event allows for acidification of BCV and expression of acidic-contingent bacterial factors, *e.g.*, *virB*; simultaneously, the cathepsin D action does not take place (Boschiroli *et al.*, 2001; Starr *et al.*, 2008; von Bargen *et al.*, 2012). *Brucella* type IV secretion (T4SS encoded by *virB* operon) is responsible for regulation of intracellular trafficking from autophagosome to endoplasmatic reticulum (ER) (Fig.1) (Gorvel and Moreno, 2002).

About 1 hour after internalization, *Brucella* organisms are located within multimembranous autophagosome with LAMP1 and Sec61β (calreculin). This structure is also called as a late BCV and occurs only in epithelial cells (Pizarro-Cerdá *et al.*, 1998). LAMP1 function is not accurately described; however, it presumably participates in the pathogen intracellular survival (Gorvel and Moreno, 2002). The final step of *Brucella* intercellular trafficking is an acquisition of the markers characteristic of the endoplasmic reticulum: calnexin, calreticulin and Sec61β, although in this step BCV lose the LAMP-1 (Pizarro-Cerdá *et al.*, 1998). However, this protein is constantly present in large vacuole only in human monocytes, in which opsonized *Brucella* multiply (Bellaire *et al.*, 2005). ER is the only compartment that is suitable for *Brucella* replication (Pizarro-Cerdá *et al.*, 1998). The mechanism of BCV-ER connection remains unclear. In this process there are involved small GTPases Rab2, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, the COPI complex (Coat Protein Complex I) and protein kinase C (PKC_I). COPI and PKC_I control vesicular trafficking to the ER from Golgi. GAPDH/COPI/Rab2/PKC_I complex is responsible for *Brucella* replication within the ER (Fugier *et al.*, 2009).

Treatment of brucellosis

Currently, there are no effective vaccines for human, although several *Brucella* vaccines are accessible for livestock. Live, attenuated vaccines bereft of virulence factors, still present residual virulence (*e.g.*, Live *B. abortus* vaccine strain 19, Live *B. abortus* vaccine strain RB51, Live *B. melitensis* vaccine strain Rev-1). Subunit vaccines are proven to be relatively safe and they raise less concerns compared to live vaccines. They do not cause infection, as they present purified proteins or DNA to stimulate immune response. Researchers are still working on the improvement in livestock vaccines and their application in preventing human infections (Yang *et al.*, 2013).

To implement a successful treatment against brucellosis, antibiotics penetrating into macrophages as well as active in acidic environment are essential (Ranjbar, 2015). Brucellosis is a disease, that rarely leads to death and responds well to diverse therapeutic strategies (Solís *et al.*, 2015). However, single-antibiotics therapy is inadequate in brucellosis, as it leads to relapse of disease (Pappas *et al.*, 2006). Similarly, the therapy with single agent like: oxytetracycline, rifampin or doxycycline, causes high rate of relapses (9–25%) and prolongation of therapy does not provide satisfying effects. The treatment with trimethoprim-sulfamethoxazole or ciprofloxacin results in relapse in 30% and 83% of cases, respectively (Ranjbar, 2015). Treatment should prevent relapse of disease, further complications (arthritis, spondylitis, sacroilitis *etc.*) and enable quick relief of symptoms. The combination of two antibiotics in therapy of infections caused by *Brucella* is more effective than monotherapy. The WHO in 1986 recommended doxycycline with rifampicin for six weeks, replaced with tetracycline in combination with streptomycin. Currently, the combinations of other antibiotics or chemotherapeutics in therapy of brucellosis are used, such as fluoroquinolones or co-trimoxazole with rifampicin, doxycycline-streptomycin and doxycycline-rifampicin (Skalsky *et al.*, 2008). During the treatment of brucellosis with streptomycin and doxycycline (SD), a failure of treatment and relapse rates at 7.4% and 4.8%, respectively were noted. Almost similar results of therapy were observed during therapy with doxycycline and rifampin (DR) or streptomycin together with tetracycline (ST); however, their relapse rates were higher than in SD treatment. Another dual therapies of brucellosis are known, for example doxycycline and gentamicin (DG) with the average failure rate of 5.2% and the relapse rate

of 5.9%, or cotrimoxazole and rifampicin (RCTM) used in children brucellosis with the failure of treatment and relapse rates at 0–16.4% and 3.1–10%, respectively. The treatment with ciprofloxacin or ofloxacin with doxycycline, cotrimoxazole, rifampicin brought about the relapse rate between 3.2 to 26% (average 11.4%) and the failure rate between 3.2% to 26% (12.2%) (Alavi and Alavi, 2013). There were three clinical trials that used triple-drug therapy with doxycycline, rifampicin and aminoglycoside. There is no clear evidence on the superiority of triple-drug therapy when compared to the two-drug therapy. Nevertheless, it seems that triple-drug therapy is more effective in preventing relapses, but less successful in short-term treatment than two-drug therapy (Solís *et al.*, 2015). The research by Alavi and Alavi (2013) suggested triple therapy for eight weeks in complicated cases (with spondylitis, or arthritis) due to lower treatment failure rates than two-drug therapy. Doxycycline and aminoglycoside therapy is recommended in uncomplicated chronic, or acute cases and in complicated cases without endocarditis, spondylitis, arthritis. In uncomplicated cases, streptomycin and doxycycline, or gentamicin are also advocated (Alavi and Alavi, 2013).

Smith and colleagues (2013) found a new strategy to treat brucellosis. The connection of BCV with the ER requires remodeling of endoplasmatic reticulum, which is necessary in modification of the ER structure during host stress response, which is called the Unfolded Protein Response (UPR). The disruption of UPR, through tauroursodeoxycholic acid drug can inhibit *Brucella* replication. UPR can be a novel target in the of brucellosis (Smith *et al.*, 2013).

There are studies concerning the influence of ginseng saponin fraction A (RGSF-A) for combating brucellosis. Ginseng is a valued plant in Asia, considered a panacea to variety range of diseases. Arayan *et al.* (2015) examined the influence of RGSF-A for eradication of bacterial infection in RAW 264.7 cells. In this study, the bacterial internalization and adhesion were reduced in the cells treated when compared to the control cells without treatment. RGSF-A takes part in downregulation of MAPKs (mitogen-activated protein kinases) and hence, limits polymerization of F-actin and inhibits bacterial penetration into the cells. RGSF-A influences also intracellular trafficking of *B. abortus* and favors interaction of *B. abortus*-containing phagosomes (BCPs) with LAMP-1. LAMP-1 is transmembrane protein, that is responsible for the fusion of lysosomes with phagosomes, enabling the connection of BCPs with lysosome and elimination of bacteria (Arayan *et al.*, 2015; Reyes *et al.*, 2016; Huy *et al.*, 2017). Huy *et al.* (2017) have proven that ginsenoside Rg3 – panaxadiol saponin components of RGSF-A have been the major factor controlling brucellosis.

Undoubtedly, there are also other promising herbal plants *e.g. Teucrium polium, Scophularia deserti, Alhagi, Eucalyptus*, garlic and roots of barberry that contain bioactive ingredients (flavones, flavonoids, anthocyanins and tanins) that can be effecive in preventing or even combat brucellosis (Naghadi *et al.*, 2016).

Conclusions

Brucella is an intracellular pathogen, especially dangerous for domestic animals, which causes massive infections and thus significant economic losses. Moreover, people who work with infected animals comprise a risk group, *e.g.*, farmers, veterinarians, or laboratorians and they are most endangered of being exposed to the pathogen. Brucellosis in human causes non-specific symptoms, therefore no plausible estimation can be managed to detect the number of infected people. *Brucella* is an inquisitive etiological agent, as does not produce classical virulence factors. The process of infection is a complex one, and there are many unexplained issues associated with it. Therefore, further studies of infection mechanisms are required.

Literature

Alavi S.M. and L. Alavi. 2013. Treatment of brucellosis: a systematic review of studies in recent twenty years. *Caspian. J. Intern. Med.* 4(2): 636–641.

Al Dahouk S., L.D. Sprague and H. Neubauer. 2013. New developments in the diagnostic procedures for zoonotic brucellosis in humans. *Rev. Sci. Tech.* 32(1): 177–188.

Alshaalan M.A., S.A. Alalola, M.A. Almuneef, E.A. Albanyan, H.H. Balkhy, D.A. AlShahrani and S. AlJohani. 2014. Brucellosis in children: Prevention, diagnosis and management guidelines for general pediatricians endorsed by the Saudi Pediatric Infectious Diseases Society (SPIDS). *Int. J. Ped. Adolesc. Med.* 1(1): 40–46.

Alton G.G. and J.R.L. Forsyth. 1996. Brucella, pp. 514–521. In: Baron S. (ed.). *Medical Microbiology*. University of Texas Medical Branch at Galveston, Galveston.

Andersen-Nissen E., K.D. Smith, K.L. Strobe, S.L. Barrett, B.T. Cookson, S.M. Logan and A. Aderem. 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. *PNAS.* 102(26): 9247–9252.

Arayan S.T., H.L. Simborio, A.W. Reyes, H.T. Hop, W. Min and H.J. Lee. 2015. The effects of red ginseng saponin fraction-A (RGSF-A) on phagocytosis and intracellular signaling in *Brucella abortus* infected RAW 264.7 cells. *FEMS Microbiol. Lett.* 362(11). doi:10.1093/femsle/fnv070.

Arellano-Reynoso B., S. Lapaque, S. Salcedo, G. Briones, A.E. Ciocchini and R. Ugadle. 2005. Cyclic-1,2-glucan is a *Brucella* virulence factor required for intracellular survival. *Nat. Immunol.* 6(6): 618–625.

Baddour M.M. and D.H. Alkhalifa. 2008. Evaluation of three polymerase chain reaction techniques for detection of Brucella DNA in peripheral human blood. *Can. J. Microbiol.* 54(5): 352–357.

Baldi P.C. and G.H. Giambartolomei. 2013. Pathogenesis and pathobiology of zoonotic brucellosis in humans. *Rev. Sci. Tech. Off. Int. Epiz.* 32(1): 117–125.

Bandara A.B., N. Sriranganathan, G.G. Schurig and S.M. Boyle. 2007. Carboxyl-terminal protease regulates *Brucella suis* morphology in culture and persistence in macrophages and mice. *J. Bacteriol.* 187(16): 5767–5775.

von Bargen K., J.P. Gorvel and S.P. Salcedo. 2012. Internal affairs: investigating the Brucella intracellular lifestyle. *FEMS Microbiol. Rev.* 36(3): 533–562.

Beck B.L., L. Tabatabi and J.E. Mayfield. 1990. A protein isolated from *Brucella abortus* is a Cu-Zn superoxide dismutase. *Biochem.* 29(2): 372–376.

Bellaire B.H., R.M. Roop II and J.A. Cardelli. 2005. Opsonized virulent *Brucella abortus* replicates within nonacidic, endoplasmic reticulum-negative, LAMP-1-positive phagosomes in human monocytes. *Infect. Immun.* 73(6): 3702–3713.

Benov L.T. and I. Fridovich. 1994. *Escherichia coli* Expresses a Copper- and Zinc-containing Superoxide Dismutase. *J. Biol. Chem.* 269(41): 25310–25314.

Bingöl A., N. Yücemen and O. Meço. 1999. Medically treated intraspinal "*Brucella*" granuloma. *Surg. Neur.* 52(6): 570–576.

Bohin J.P. 2000. Osmoregulated periplasmic glucans in Proteobacteria. *FEMS Misrobiol. Lett.* 186(1): 11–19.

Boschiroli M.L., V. Foulongne and D. O'Callaghan. 2011. Brucellosis: a worldwide zoonosis. *Curr. Opin. Microbiol.* 4: 58–64.

Brown D.A. and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell. Dev. Biol.* 14: 111–136.

Byndloss M.X. and R.M. Tsolis. 2016. *Brucella* spp. virulence factors and immunity. *Annu. Rev. Anim. Biosci.* 4: 111–127.

O'Callaghan D., C. Cazevieille, A. Allardet-Servent, M.L. Boschiroli, G. Bourg, V. Foulongne, P. Frutos, Y. Kulakov and M. Ramuz. 1999. A homologue of the Agrobacterium tumefaciens VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of Brucella suis. *Mol. Microbiol.* 33(6): 1210–1220.

Cardosos P.G., G.C. Macedo, V. Azevedo and S.C. Oliveira. 2006. *Brucella* spp. noncanonical LPS: structure, biosynthesis, and interaction with host immune system. *Microb. Cell Fact.* 5: 13. doi:10.1186/1475-2859-5-13.

Carvalho Neta A.V., A.P.R. Stynen, T.A. Paixão, K.L. Miranda, F.L. Silva, C.M. Roux, R.M. Tsolis, R.E. Everts, H.A. Lewin, L.G. Adams and others. 2008. Modulation of bovine trophoblastic innate immune response by *Brucella abortus. Infect. Immun.*76(5): 1897–1907.

Carvalho Neta A.V., J.P. Mol, M.N. Xavier, T.A. Paixão, A.P. Lage and R.L. Santos. 2010. Pathogenesis of bovine brucellosis. *Vet. J.* 184(2): 146–155.

Cascales E. and P.J. Christie. 2003. The versatile bacterial type IV secretion system. *Nat. Rev. Microbiol.* 1(2): 137–149.

Celli J., C. de Chastelier, D.M. Franchini, J. Pizzaro-Cerda, E. Moreno and J.P. Gorvel. 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. *J. Exp. Med.* 198(4): 545–556.

Celli J. 2006. Surviving inside a macrophage: the many ways of Brucella. *Res. Microbiol.* 157: 93–98.

Chaudhary A., K. Ganguly, S. Cabantous, G.S. Waldo, S.N. Micheva-Viteva, K. Nag, W.S. Hlavacek and C.S. Tung. 2012. The Brucella TIR-like protein TcpB interacts with the death domain of MyD88. *Biochem. Biophys. Res. Commun.* 417(1): 299–304.

Chen L., Q.W. Xie and C. Nathan. 1998. Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. *Mol. Cell.* 1(6): 795–805.

Christopher S., B.L. Umapathy and K.L. Ravikumar. 2010. Brucellosis: Review on the recent trends in pathogenicity and laboratory diagnosis. *J. Lab. Physicians.* 2(2): 55–60.

Coelho A.C., J.G. Díez and A.M. Coelho. 2015. Risk Factors for *Brucella* spp. in Domestic and Wild Animals, pp. 2–31. In: Baddour M (ed.). *Updates on Brucellosis*. InTech.

Corbel M.J. 1997. Brucellosis: an overview. *Emerg. Infect. Dis.* 3(2): 213–221.

Delure R.M., M. Martinez-Lorenzo, P. Lestrate, I. Danese, V. Bielarz, P. Mertens, X. De Bolle, A. Tibor, J.P. Gorvel and J.J. Letesson. 2001. Identification of *Brucella* spp. genes involved in intracellular trafficking. *Cell Microbiol.* 3(7): 487–497.

Del Vecchio V.G., V. Kapatral, R.J. Redkar, G. Patra, C. Mujer, T. Los, N. Ivanova, I. Anderson, A. Bhattacharyya, A. Lykidis and others. 2002. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. *Proc. Natl. Acad. Sci. USA* 99(1): 443–448.

Dwight D. and M.S. Bowman. 2011. Introduction to the Alphaproteobacteria: *Wolbachia* and *Bartonella*, *Rickettsia*, *Brucella*, *Ehrlichia* and *Anaplasma. Top. Companion Anim. Med.* 26(4): 173–177.

Endley S., D. McMurray and T.A. Ficht. 2001. Interruption of the *cydB* locus in *Brucella abortus* attenuates intracellular survival and virulence in the mouse model of infection. *J. Bacteriol.* 183(8): 2454–2462.

Fernandez-Prada C.M., E.B. Zelazowska, M. Nikolich, T. Hadfield, R.M. Roop II, G.L. Robertson and D.L. Hoover. 2003. Interactions between *Brucella melitensis* and human phagocytes: Bacterial surface O-polysaccharide inhibits phagocytosis, bacterial killing, and subsequent host cell apoptosis. *Infect. Immunity.* 71(4): 2110–2119.

de Figueiredo P., T.A. Ficht, A. Rice-Ficht, C.A. Rossetti and L.G. Adams. 2015. Pathogenesis and immunobiology of brucellosis review of *Brucellae* host interactions. *Am. J. Pathol.* 185(6): 1505–1517.

Franco M.P., M. Mulder, R.H. Gilman and H.L. Smits. 2007. Human brucellosis. *Lancet. Infect. Dis.* 7(12): 775–786.

Fugier E., S.P. Salcedo, C. de Chastellier, M. Pophillat, A. Muller, V. Arce-Gorvel, P. Fourquet and J.P. Gorvel. 2009. The glyceraldehyde-3-phosphate dehydrogenase and the small GTPase Rab 2 are crucial for *Brucella* replication. *PLoS Path.* 5(6):e1000487. doi:10.1371/journal.ppat.1000487.

Galińska E.M. and J. Zagórski. 2013. Brucellosis in humans – etiology, diagnostics, clinical forms. *Ann. Agric. Environ. Med.* 20(2): 233–238.

Gee J.M., M.E. Kovach, V.K. Grippe, S. Hagius, J.V. Walker, P.H. Elzer and others. 2004. Role of catalase in the virulence of Brucella melitensis in pregnant goats. *Vet. Microbiol.* 102(1–2): 111–115.

Gee J.M., M.E. Kovach, V.K. Grippe, S. Hagius, J.V. Walker, P.H. Elzer and R.M. Roop 2nd. 2005. The *Brucella abortus* Cu, Zn superoxide dismutase is required for optimal resistance to oxidative killing by murine macrophages and wild-type virulence in experimentally infected mice. *Infect. Immun.* 73(5): 2873–2880.

Gerasu M.A. and G.M. Kassa. 2016. A review on diagnostic methods of brucellosis. *Vet. Sci. Tech.* 7:3. doi:10.4172/2157-7579.1000323.

Godfroid J., B. Garin-Bastuji, C. Saegerman and J.M. Blasco. 2013. Brucellosis in terrestrial wildlife. *Rev. Sci. Tech.* 32(1): 27–42.

Gomez G., L.G. Adams, A. Rice-Ficht and T.A. Ficht. 2013. Host-*Brucella* interactions and the *Brucella* genome as tools for subunit antigen discovery and immunization against brucellosis. *Front. Cell. Infect. Microbiol.* 3:17. doi:10.3389/fcimb.2013.00017.

Gopal R.K. and S. Elumalai. 2017. Industrial production of Superoxide Dismutase (SOD): A mini review. *J. Probe. Health.* 5:3. doi:10.4172/2329-8901.1000179.

Gorvel J. and E. Moreno. 2002. *Brucella* intracellular life: from invasion to intracellular replication. *Vet. Microbiol.* 90(1–4): 281–297.

Gupte S. and T. Kaur. 2015. Diagnosis of Human Brucellosis. *J. Top. Dis.* 4:1. doi:10.4185/2329-891X.1000185.

Guzmán-Verri C., E. Chaves-Olarte, C. von Eichel-Streiber, I. López-Goñi, M. Thelestam, S. Arvidson, J.P. Gorvel and E. Moreno. 2001. GTPases of the Rho subfamily are required for *Brucella abortus* internalization in nonprofessional phagocytes. *J. Biol. Chem.* 276(48): 44435–44443.

Gwida M., S. Al Dahouk, F. Melzer, U. Rősler, H. Neubauer and H. Tomaso. 2010. Brucellosis – regionally emerging zoonotic disease? *Croat. Med. J.*51(4): 289–295.

He Y. 2012. Analyses of Brucella pathogenesis, host immunity, and vaccine targets using systems biology and bioinformatics. *Front. Cell. Infect. Microbiol.* 2:2. doi:10.3389/fcimb.2012.00002.

Huy T.X., A.W. Reyes, H.T. Hop, L.T. Arayan, W. Min and H.J. Lee. 2017. Intracellular trafficking modulation by ginsenoside Rg3 Inhibits *Brucella abortus* uptake and intracellular survival within RAW 264.7 cells. *J. Microbiol. Biotechnol.* 27(3): 616–623.

Iowa State University. The Centerfor Food Security & Public Health. 2009. Ovine and Caprine Brucellosis: *Brucella melitensis*. http://www.cfsph.iastate.edu/Factsheets/pdfs/brucellosis_melitensis.pdf.

Kagan J.C. and R. Medzhitov. 2006. Phosphoinositide-mediated adaptor recruitment controls toll-like receptor signaling. *Cell.* 125(5): 943–955.

Kim S. 2015. The interaction between *Brucella* and the host cell in phagocytosis, pp. 45–60. In: Baddour M. (ed.). *Updates on Brucellosis*. InTech, Jinju.

Kőhler S., V. Foulongne, S. Ouahrani-Bettache, G. Bourg, J. Teyssler, M. Ramuz and J.P. Liautard. 2002. The analysis of the intramacrophagic virulome of *Brucella suis* deciphers the environment encountered by the pathogen inside the macrophage host cell. *Proc. Natl. Acad. Sci. USA*. 99(24): 15711–15716.

Kusumawati A., C. Cazevieille, F. Porte , S. Bettache, J.P. Liautard and J. Widada. 2000. Early events and implication of F-actin and annexin I associated structures in the phagocytic uptake of *Brucella suis* by the J-774A.1 murine cell line and human monocytes. *Microb. Pathog.* 28(6): 343–352.

Lapaque N., I. Moriyon, E. Moreno and J.P. Gorvel. 2005. *Brucella* lipopolysaccharide acts as a virulence factor. *Curr Opin Microbiol.* 8(1): 60–66.

Lavigne J.P., G. Petey, F.J. Sangari, G. Bourg, M. Ramuz, D. O'Callaghan, S. Michaux-Charachon. 2005. Identification of a new virulence factor, BvfA, in *Brucella suis. Infect. Immun.* 73(9): 5524–5529.

Loiser-Meyer S., M.P.J. de Bagüés, S. Köhler, J.P. Liautard and V. Jubier-Maurin. 2005. Differential use of the two high-oxygen-affinity terminal oxidases of *Brucella suis* for *in vitro* and intramacrophagic multiplication. *Infect. Immun.* 73(11): 7768–7771.

López-Goñi I., C. Guzmán-Verri, L. Manterola, A. Sola-Landa, I. Moriyón, E. Moreno. 2002. Regulation of *Brucella* virulence by the two – component system BvrR/BvrS. *Vet. Microbiol.* 90(1–4): 329–339.

Manterola L., C. Guzmán-Verri, E. Chaves-Olarte, E. Barquero-Calvo, M.J. de Miguel, I. Moriyón, M.J. Grilló, I. López-Goñi and E. Moreno. 2007. BvrR/BvrS-Controlled Outer Membrane Proteins Omp3a and Omp3b are not essential for *Brucella abortus* virulence. *Infect. Immun.* 75(10): 4867–4874.

Martin D.W., J.E. Baumgartner, J.M. Gee, E.S. Anderson and R.M. Roop II. 2012. SodA is a major metabolic antioxidant in Brucella abortus 2308 that plays a significant, but limited, role in the virulence of this strain in the mouse model. *Microbiology.* 158(Pt7): 1767–1774.

Martínez-Nũñez C., P. Altamirano-Silva, F. Alvarado-Guillén, E. Moreno, C. Guzmán-Verri and E. Chaves-Olarte. 2010. The Two-Component System BvrR/BvrS regulates the expression of the type IV secretion system VirB in *Brucella abortus*. *J. Bacteriol.* 192(21): 5603–5608.

Megid J., L.A. Mathias and C.A. Robles. 2010. Clinical manifestations of brucellosis in domestic animals and humans. *Op. Vet. Sci. J.* 4: 119–126.

Mizak L., R. Gryko, S. Parasion and M. Kwiatek. 2014. Brucellosis – a worldwide zoonosis (in Polish). *Życ. Wet.* 89(1): 35–40.

Mizuno T. and I. Tanaka. 1997. Structure of the DNA-binding domain of the OmpR family of response regulators. *Mol. Microbiol.* 24(3): 665–667.

Mol J.P.S., E.A. Costa, A.F. Carvalho, Y-H. Sun, R.M. Tsolis, T.A. Paixão and R.L. Santos. 1995. Molecular biology of microbial ureases. *Microbiol. Rev.* 59(3): 451–480.

Mol J.P.S., E.A. Costa, A.F. Carvalho, Y-H. Sun, R.M. Tsolis, T.A. Paixão and others. 2014. Early transcriptional responses of bovine chorioallantoic membrane explants to wild type, Δ*virB2* or Δ*btpB Brucella abortus* infection. *PLoS One.* 9(9): e108606. doi:10.1371/journal.pone.0108606.

Naghadi N., H. Assanzad-Azar and A. Delpisheh. 2016. The most important medicinal plants for treatment of brucellosis. *J. Prev. Epi.* 1(2): e20.

Naroeni A. and F. Porte. 2002. Role of Cholesterol and the Ganglioside GM 1 in entry and short-term survival of *Brucella suis* in murine macrophages. *Infect. Immun.* 70(3): 1640–1644.

Newman R.M., P. Salunkhe, A. Godzik and J.C. Reed. 2006. Identification and characterization of a novel bacterial virulence factor that shares homology with mammalian toll/interleukin-1 receptor family proteins. *Infect. Immun.* 74(1): 594–601.

Oliveira S.C., F.S. de Oliviera, G.C. Macedo, L.A. de Almeida and N.B. Carvalho. 2008. The role of innate immune receptors in the control of *Brucella abortus* infection: Toll-like receptors and beyond. *Microbes Infect.* 10(9): 1005–1009.

Osman A.Y., F.F. Jesse, A. Abdul Kadir and A.A. Saharee. 2016. The epidemiology and immunopathophysiology of brucellosis in small ruminant. *PJSRR.* 2(1): 11–21.

Pappas G., P. Papadimitriou, N. Akritidis, L. Christou and E. Tsianos. 2006. The new global map of human brucellosis. *Lancet. Infect. Dis.* 6(2): 91–99.

Perkins S.D., S.J. Smither and H.S. Atkins. 2010. Towards a *Brucella* vaccine for humans. *FEMS Microbiol. Rev.* 34: 379–394.

Pizarro-Cerdá J., E. Moreno and J.P. Gorvel. 2000. Invasion and intracellular trafficking of *Brucella abortus* in nonphagocytic cells. *Microbes Infect.* 2(7): 829–835.

Pizarro-Cerdá J., S. Méresse, R.G. Parton, G. van der Goot, A. Sola-Landa, I. López-Goñi, E. Moreno and J.P. Gorvel. 1998. *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. *Infect. Immun.* 66(12): 5711–5724.

Porte F., A. Naroeni, S.Ouahrani-Bettache and J.P. Liautard. 2003. Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome fusion in murine macrophages. *Infect. Immun.* 71(3): 1481–1490.

Radhakrishnan G.K., Q. Yu , J.S. Harms and G.A. Splitter. 2009. Brucella TIR domain-containing protein mimics properties of the toll-like receptor adaptor protein TIRAP. *J. Biol. Chem*. 284(15): 9892–9898.

Ranjbar M. 2015. Treatment of brucellosis, pp. 171-184. In: Baddour M. (ed.). *Updates on Brucellosis*. InTech, Teheran.

Reyes A.W., H.L. Simborio, H.T. Hop, L.T. Arayan, W.G. Min, H.J. Lee, M.H. Rhee, H.H. Chang and S. Kim. 2016. Inhibitory effect of red ginseng acidic polysaccharide from Korean red ginseng on phagocytic activity and intracellular replication of Brucella abortus in RAW 264.7 cells. *J. Vet. Sci.* 17(3): 315–321.

Rubach M.P., J.E. Halliday, S. Cleaveland and J.A. Crump. 2013. Brucellosis in low-income and middle-income countries. *Curr. Opin. Infect. Dis.* 26(5): 404–412.

Salcedo S.P., M.I. Marchesini, C. Degos, M. Terwagne, K. Von Bargen, H. Lepidi, C.K. Hermann, T.L. Santos Lacerda, P.R. Imbert, P. Pierre and others. 2013. BtpB, a novel Brucella TIR-containing effector protein with immune modulatory functions. *Front. Cell. Infect. Microbiol*. 3:28. doi:10.3389/fcimb.2013.00028.

Sangari F.J. and J. Agűero. 1996. Molecular basis of *Brucella* pathogenicity: an update. *Microbiologia.* 12(2): 207–218.

Sangari F.J., A. Seoane, M.C. Rodriguez, J. Agüero and J.M. Garcia Lobo. 2007. characterization of the urease operon of Brucella

abortus and assessment of its role in virulence of the bacterium. *Infect. Immun.* 75(2): 774–780.

Scholz H.C., S. Revilla-Fernández, S. Al Dahouk, J.A. Hammerl, M.S. Zygmunt, A. Cloeckaert, M. Koylass, A.M. Whatmore, J. Blom, G. Vergnaut and others. 2016. *Brucella vulpis* sp. nov., isolated from mandibular lymph nodes of red foxes (*Vulpes vulpes*). *Int. J. Syst. Evol. Microbiol.* 66(5): 2090–2098.

Seleem M.N., S.M. Boyle and N. Sriranganathan. 2008. *Brucella*: A pathogen without classic virulence genes. *Vet. Microbiol.* 129(1–2): 1–14.

Sengupta D., A. Koblansky, J. Gaines, T. Brown, A.P. West and D. Zhang. 2010. Subversion of innate immune responses by *Brucella* through the targeted degradation of the TLR signaling adapter, MAL. *J. Immunol.* 184(2): 956–964.

Skalsky K., D. Yahav, J. Bishara, S. Pitlik, L. Leibovici and M. Paul. 2008. Treatment of human brucellosis: systematic review and meta-analysis of randomised controlled trials. *BMJ*. 336(7646): 701–704.

Smith J.A., M. Khan, D.D. Magnani, J.S. Harms, M. Durward, G.K. Radhakrishnan. 2013. *Brucella* induces an unfolded protein response via TcpB that supports intracellular replication in macrophages. *PLoS Path.* 9(12): e1003785. doi:10.1371/journal.ppat.1003785.

Sola-Landa A., J. Pizarro-Cerdá, M.J. Grilló, E. Moreno, I. Moriyón and J.M. Blasco. 1998. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. *Mol. Microbiol.* 29(1): 125–138.

Solís J., G. del Pozo and J. Solera. 2015. Treatment of human brucellosis-review of evidence from clinical trials, pp. 186–189. In: Baddour M. (eds). *Updates on Brucellosis.* InTech, Villarrobledo, Albacete.

Starr T., T.W. Ng, T.D. Wehrly, L.A. Knodler and J. Celli. 2008. *Brucella* intracellular replication requires trafficking through the late endosomal/lysosomal compartment. *Traffic.* 9(5): 678–694.

Stevanin T.M., J.W.B. Moir and R.C. Read. 2005. Nitric oxide detoxification systems enhance survival of *Neisseria meningitidis* in human macrophages and in nasopharyngeal mucosa. *Infect. Immun.* 73(6): 3322–3329.

Stock J.B., M.G. Surette, M. Levit and P. Park. 1995. Two-component siganl transduction systems: structure-function relationships and mechanism of catalysis, pp. 25–51. In: Hoch J.A. and T.J. Silhavy (eds). *Two Component Signal Transduction.* ASM Press, Washington, D.C.

Tan K.K., Y.C.Tan, L.Y. Chang, K.W. Lee, S.S. Nore, W.Y. Yee, M.N.M. Isa, F.L. Jafar, C.C. Hoh and S. AbuBakar. 2015. Full genome SNP-based phylogenetic analysis reveals the origin and global spread of Brucella melitensis. *BMC Genomics.* 16(1):93. doi:10.1186/s12864-015-1294-x.

Uematsu S. and S. Akira. 2008. Toll-Like Receptors (TLRs) and their ligands. *Handb. Exp. Pharmacol.* (183): 1–20.

Waterman-Storer C.M., R.A. Worthylake, B.P. Liu, K. Burridge and E.D. Salmon. 1999. Microtubule growth activates Rac1 to promote lamellipodial protrusion in fibroblasts. *Nat. Cell. Biol.* 1(1): 45–50.

Whatmore A.M., N. Davison, A. Cloeckaert, S. Al Dahouk, M.S. Zygmunt, S.D. Brew, L.L. Perrett, M.S. Koylass, G. Vergnaud, C. Quance and others. 2014. *Brucella papionis* sp. nov., isolated from baboons (*Papio* spp.). *Int. J. Syst. Evol. Microbiol.* 64(Pt12): 4120–4128.

Viadas C., M.C. Rodríguez, F.J. Sangari, J.P. Gorvel, J.M. García-Lobo and I. López-Goñi. 2010. Transcriptome *Analysis* of the *Brucella abortus* BvrR/BvrS *Two-Component Regulatory System*. *PLoS ONE*. 5(4): e10216. doi:10.1371/journal.pone.0010216.

Xavier M.N., T.A. Paixao, A.B. den Hartigh, R.M. Tsolis and R.L. Santos. 2010. Pathogenesis of *Brucella* spp. *Op. Vet. Sci. J.* 4: 109–118.

Yang X., J.A. Skyberg, L. Cao, B. Clapp, T. Thornburg and D.W. Pascual. 2013. Progress in *Brucella* vaccine development. *Front. Biol.* 8(1): 60–77.

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ORIGINAL PAPER

Isolation of Bacteriocin-producing *Staphylococcus* spp. Strains from Human Skin Wounds, Soft Tissue Infections and Bovine Mastitis

MAGDALENA ZALEWSKA[1, 3], JOHN J. CHUREY[2], RANDY W. WOROBO[3], SŁAWOMIR MILEWSKI[1], PIOTR SZWEDA[1]*

[1] Gdansk University of Technology, Department of Pharmaceutical Technology and Biochemistry, Gdansk, Poland
[2] Cornell University, Department of Food Science, New York State Agricultural Experiment Station, Geneva, USA
[3] Cornell University, Department of Food Science, Ithaca, USA

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Abstract

A collection of 206 *Staphylococcus* spp. isolates was investigated for their ability to produce compounds exhibiting antistaphylococcal activity. This group included *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus xylosus* strains recovered from bovine mastitis (n = 158) and human skin wounds and soft tissues infections (n = 48). Production of substances with antimicrobial activity was observed in six strains. Five of them were recovered from bovine mastitis, and one was isolated from the infected human skin wound. Three of the six antimicrobials produced by the different strains showed substantial loss of antimicrobial activity upon treatment with proteolytic enzymes, which suggests their peptidic structure. Additional studies have shown that one of the putative bacteriocins was efficiently secreted to the liquid medium, facilitating its large-scale production and isolation. The peptide produced by the M2B strain exhibited promising activity; however, against narrow spectrum of *Staphylococcus* spp. clinical and animal isolates. Growth inhibition was observed only in the case of 13 (including nine *S. aureus*, three *S. xylosus* and one *S. epidermidis* strains) out of 206 strains tested. Important advantage of the produced agent was its high thermal stability. Fifteen minutes of incubation at 90°C did not affect its antimicrobial potential. The highest efficiency of production of the agent was demonstrated in TSB medium after 24 hours at 37°C. The researches revealed that ability to production of bacteriocin among staphylococci is not very common. Only one (*S. xylosus* strain assigned as M2B) out of 206 strains tested produced satisfactory amounts of antistaphylococcal bacteriocin. In spite of that, we would encourage other researchers for investigation of their collections of *Staphylococcus* spp. isolates towards selection strains producing antimicrobial agents.

Key words: antimicrobial peptides, antistaphylococcal agents, *Staphylococcus*, *Staphylococcus aureus*, *Staphylococcus xylosus*

Introduction

Bacterial cells may produce several types of substances that inhibit the growth of other microorganisms. These antimicrobial agents belong to different classes of molecules, ranging from the simple compounds, like hydrogen peroxide or lactic acid to more complicated: antibiotics, peptides, exotoxins or lytic enzymes (Brito *et al.*, 2011). Among them, the agents of proteinaceous nature, peptides and proteins, seem to be especially interesting. Bacteria produce two essential classes of products of this type: bacteriocins and peptide antibiotics. Both of them are secreted to help the host strain to eliminate other microorganisms from the place of its growth. However, there are some important differences between these two classes of agents. First of all, enzymes synthesize the peptide antibiotics, whilst the bacteriocins are ribosomally synthesized. Bacteriocins are targeted at a narrow spectrum of bacteria often within the species of the producer or closely related ones, while the classical antibiotics are active against broad spectra of bacteria. Another important feature that separates bacteriocins from antibiotics is their potency against susceptible bacteria; bacteriocins are unique because they can kill bacteria at nanomolar concentrations, while antibiotics are needed in much higher concentrations (Nes, 2011). The bacteriocins are included in the larger group of antimicrobial substances defined as antimicrobial peptides (AMPs). These peptides are ribosomally synthesized by all domains of living organisms (eukaryotes, bacteria and archaea). Similarly as bacteria the other organisms (*e.g.* plants,

* Corresponding author: P. Szweda, Gdansk University of Technology, Department of Pharmaceutical Technology and Biochemistry, Gdansk, Poland; **e-mail: piotr.szweda@wp.pl**

animals and humans) produce AMPs for elimination of pathogenic microorganisms and prevention of infections (AMPs constitute an important element of their innate immune system) (Rashid *et al*., 2016). AMPs such as human defensins, human neutrophil peptides and many bacterial bacteriocins are cationic and are able to bind to anionic parts of bacterial surface (*e.g.* anionic lipids or other anionic components) (Joerger, 2003). AMP's could undergo posttranslational modification *e.g.* proteolytic processing or glycosylation (Rashid *et al*., 2016).

The modes of action of AMPs, including most of the bacteriocins, involve interactions with the bacterial cell membrane, and forming complexes with lipid II – a highly conserved precursor of the bacterial cell wall (Malanovic and Lohner, 2016). Cell membrane permeabilization leads to efflux of important intracellular molecules such as mono- and divalent ions (K^+, Na^+, Ca^{2+} and Mg^{2+}) and ATP (Di Meo *et al.,* 2016; Yeaman and Yount, 2003). Binding the peptides with lipid II inhibits peptidoglycan synthesis and additionally promotes pore formation and membrane disruption (Malanovic and Lohner, 2016). Structure-activity analyses of a broad range of peptides showed two main requirements for their antimicrobial activity: (1) a cationic charge and (2) an induced amphipathic conformation. The diversity of lipids among pathogens could explain the differences in activity of a single peptide between different types of microorganisms (Powers and Hancock, 2003).

Due to high selectivity and activity against a substantial array of pathogenic microorganisms, AMP's are a promising alternative to conventional antibacterial antibiotics (Kosikowska and Lesner, 2016; Ołdak and Zielińska, 2017). Compared to conventional antibiotic therapy, killing of microorganisms by peptides is more rapid and they can 'attack' multiple molecular targets related to membrane and cell wall, which highly prevents development of resistant strains. However, the results of recent years revealed that many important Gram-positive as well as Gram-negative bacteria, including dangerous human and animal pathogens, have developed mechanisms of modification of most important molecular targets of AMPs, namely cell membrane and lipid II. As a consequence these strains are highly resistant to AMPs' activity (Andersson *et al.,* 2016).

In the present study, a collection of *Staphylococcus* spp. strains isolated from human skin wounds, soft tissues infections, as well as from milk of bovine suffering from mastitis (*S. aureus*, *S. epidermidis*, *S. xylosus*) was screened for their ability to produce antibacterials, particularly with proteinaceous structure, with activity against other staphylococci. This research was focused on identification and preliminary characterization of the newly discovered antimicrobial peptides in order to uncover their potential therapeutic application in medicine and veterinary science for elimination of pathogenic bacteria from food products and also possibilities of their large-scale production.

Experimental

Materials and methods

Bacterial strains. *Staphylococcus* spp. strains (n = 206) were isolated from milk of bovine suffering from mastitis (n = 158) (Jakubczak *et al*., 2007; Kot *et al*., 2012) and from human skin wounds and soft tissue infections (n = 48). Eighteen of the human derived isolates (collected in 2013) were supplied by the Microbial Laboratory at the Provincial Hospital, Koszalin (Poland) and thirty strains were obtained from the Department of Clinical Microbiology, Central Hospital, Vaxjo (Sweden, collected between November 2006 and April 2007) (Sjölund and Kahlmeter, 2008). All isolates of human origin were classified as *S. aureus* (n = 48) and strains isolated from milk belonged to three species: *S. aureus* (n = 120), *S. epidermidis* (n = 16) and *S. xylosus* (n = 22). The strains isolated from mastitis were collected in two periods of time: between January 2005 and December 2006 (90 isolates of *S. aureus*) and between February 2009 and March 2010 (all *S. epidermidis* and *S. xylosus* and remaining *S. aureus*). Additionally *Listeria monocytogenes* and *Micrococcus luteus* strains were used as indicatory strains in antimicrobial activity assays. All strains were grown in liquid Tryptic Soy Broth – TSB (Fluka) and on solid Tryptic Soy Agar – TSA (Tryptic Soy Broth + 1.5% agar (Sigma)). The strains were stored at –70°C as a cell suspension in PBS buffer (Phosphate Buffered Saline, pH 7.4; Sigma) containing 40% glycerol (vol/vol).

Detection of AMP production. Production of antimicrobial substances by staphylococcal cells was evaluated by the overlay method (Barefoot and Klaenhammer, 1983; Fleming *et al*., 1975). The single colony (obtained by streaking on TSA agar plates) of the producing strains was transferred with a sterile needle or toothpick on fresh TSA agar plates. After 24 hours of incubation at 37°C, the plates with a single, centrally located, colony – spot of potential producer strains were overlaid with soft TSA medium (Triptic Soy Broth supplemented with 0.75% Agar) inoculated with cells of the sensitive (indicator) strain to a final concentration of 0.01% (vol/vol). Volume of the soft agar depended on the size of the plate, where soft agar should completely cover the surface of the bottom TSA medium and the producer strain colony. The indicator strains (n = 7) included six staphylococci strains isolated from animal infections (1 – *S. xylosus*, 1 – *S. epidermidis* and 4 – *S. aureus*) and one strain of *Listeria monocytogenes* ATCC10425

Table I
The indicator strains used for preliminary selections of staphylococci producing antibacterial agents.

Strain	Source of the strain	Resistance
1 *S. xylosus* 30c1	Bovine mastitis	ND
2 *S. epidermidis* 267	Bovine mastitis	ND
3 *S. aureus* K10	Human skin wound (Koszalin)	Susceptible
4 *S. aureus* 11	Bovine mastitis	P, AMP, AML, S, AMC*
5 *S. aureus* 27	Bovine mastitis	N, MY, S, E, DA*
6 *S. aureus* 101 (MRSA)	Bovine mastitis	P+DA+E+MY+S+N/OX*
7 *L. monocytogenes* ATCC10425	Reference strain	ND

* – Resistance according to *Szweda et al.* 2014

(Table I). After inoculation, the plates were incubated at 37°C for another 24 h, and the growth inhibition of the indicator cells was determined. The presence of a clear zone around the producer strain colony confirmed production of an active antimicrobial agent. The diameter of the growth inhibitory zone was measured.

Antibacterial activity of the after-culture liquid medium was measured by the agar-well diffusion method (Schillinger and Lücke, 1989). The strains found as producers of antimicrobial agents on TSA agar plates (n = 6) were grown in TSB for 24 hours at 37°C. The obtained cell suspensions were centrifuged (14 000 rpm, 10 min, room temperature) to collect the bacterial cells. The collected and filter sterilized (0.22 μm) supernatants (300 μl) were aliquoted into wells (diameter 7 mm) in solid TSA medium (TSB medium supplemented with 1.5% agar). The plates were dried in a laminar flow cabinet, and when the supernatant evaporated completely from the wells, the plates were overlaid with a soft agar layer inoculated with the cells of indicator strains to a final concentration of 0.01% (vol/vol). After incubation (24 h, 37°C), the diameters of clear zones were measured.

Identification of the antimicrobials with proteinaceus nature. The overlay method (described above, section – Detection of AMP production) was also used for identification of proteinaceus nature of antimicrobials produced by the selected strains. The 2 μl of solution of proteolytic enzymes (Pronase E (20 mg/ml), α-Chymotrypsin (25 mg/ml) and Proteinase K (20 mg/ml) (Lee *et al.*, 2008)) were loaded on the surface of the bottom agar medium close to the growing colony of the producing strain – on the suspected border line of inhibition growth zone of indicator strain. The prediction of the diameter of the growth inhibition zone was based on diameters of inhibition area from the results of previous analyses. The changing of the shape of growth inhibition zone – formation of so-called "half moon zone" near the colony of producing strain confirmed susceptibility of the produced agent to the proteolytic enzymes and that the secreted product had the proteinaceus nature.

Determination of the activity spectrum of the identified AMPs. Determination of the potential antimicrobial peptide activity spectrum was performed by the overlay method (as used in the previous tests, section – Detection of AMP production) for the producer strains, which showed protease sensitivity (n = 3). All of the isolates from the collection of Department of Pharmaceutical Technology and Biochemistry, Gdansk University of Technology (n = 206) were used to define their susceptibility to the potential antimicrobial peptide. The most sensitive strain (n = 1) was selected for investigation of the antimicrobial activity in further tests, *e.g.* the optimization of production or determination of thermostability.

Optimization of AMP production parameters. The conditions for the most effective production of the bacteriocins by the strains selected in the screening procedure were optimized in terms of: (1) medium type (Tryptic Soy Broth (TSB; DB Bioscience), Mueller Hinton Broth (MHB; DB Bioscience), Brain Heart Infusion (BHI; DB Bioscience), Casamino acids (CAA; DB Bioscience) and Luria-Bertani (LB; BD Bioscience); (2) temperature (30°C, 35°C, 37°C), and (3) the growth period (12 h, 24 h and 48 h, 72 h). The cells were grown in suspension cultures with shaking (200 rpm). Samples of the cell suspensions were collected at time intervals (0 h, 3 h, 6 h, 9 h, 12 h, 24 h, 48 h, 72 h), centrifuged (10 000 rpm, 10 min, 4°C) and the activity of the supernatants obtained was determined by the well – overlay method. The first step of the procedure was performed for all (n = 3) producer strains, which exhibited sensitivity to proteolytic enzymes (*S. xylosus* M2B, *S. epidermidis* 30c1 and *S. aureus* K1). After that step, we found out that only one antimicrobial peptide, produced by strain named M2B (*S. xylosus*), was efficiently secreted to liquid medium. Thus, other tests were performed only for this strain.

Determination of thermostability. Sample of the supernatant collected from the cultures after 24 h of antimicrobial-producing strain were incubated at 25°C, 50°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C for different

time periods (5 min, 10 min, 15 min, 30 min) and their activity was determined by the well-overlay method.

Identification of the strain. Identification of the producing strain M2B was carried out by sequencing of 16S rRNA gene. PCR amplification of the target gene was carried out with a pair of primers:
rP1 5' CCCGGGATCCAAGCTTAGAGTTTGATCCTGGCTCAG 3'
fD2 5' CCGAATTCGTCGACAACACGGCTACCTTGTTACGACTT 3'
following the method described by the group of Weisburg (1991). Sequencing of the amplified product was carried out by Macrogen (The Netherlands). The analysis of sequence was performed with using MEGA 6.1 (Molecular Evolutionary Genetics Analysis) software.

Results

Screening of the 206 bacterial isolates resulted in the identification of six strains producing antistaphylococcal agents. Three producing strains were *S. aureus* isolated from bovine mastitis (assigned in the collection as 30, 31 and 32), one was *S. aureus* recovered from human skin wound (K1), one agent was produced by *S. xylosus* M2B and the last of producing strain was *S. epidermidis* 30c1. The antibacterial activity of three (produced by *S. aureus* 30, 31 and 32) out of six substances was not affected by the treatment with proteolytic enzymes, suggesting their non-proteinaceous structure. Further tests eliminated two additional strains (K1 and 30c1), since they produced antimicrobial substances exclusively when grown on solid (agar) medium while no antibacterial activity was detected in the supernatants collected from the suspension cultures. Therefore, completion of the screening procedure resulted in the identification of one strain effectively producing the antibacterial substance with protease-sensitive structure, i.e. potential bacteriocin or peptide antibiotic. This strain (M2B) and the produced agent were the subjects of further analysis.

Based on the sequence of the gene coding for 16S rRNA, the producing strain was identified as *S. xylosus* (99,9% of identity of this gene sequence with the sequences of 16S rRNA genes of other bacteria of the this genus) – labeled in the collection as M2B. The antimicrobial agent synthesized by this strain was efficiently produced both on solid as well as in a liquid medium (Fig. 1a and Fig. 2) and its' activity was eliminated in the presence of proteases (Fig. 3).

The antimicrobial spectrum of the putative antimicrobial peptide produced by *S. xylosus* M2B was determined by the well overlay method. Growth inhibition zones were observed with 13 strains of staphylococci (including nine *S. aureus*, three *S. xylosus* and one *S. epidermidis* strains), *L. monocytogenes* and *M. luteus* (Table II). Diameters of the growth inhibitory zones were in the range of 12–25 mm; surprisingly apparent substantial differences in sensitivity to the putative AMPs were found among the different *S. aureus* strains.

Conditions for the effective production of the putative bacteriocin by *S. xylosus* M2B in a liquid culture were optimized in the terms of medium type, time period and incubation temperature. Comparison of

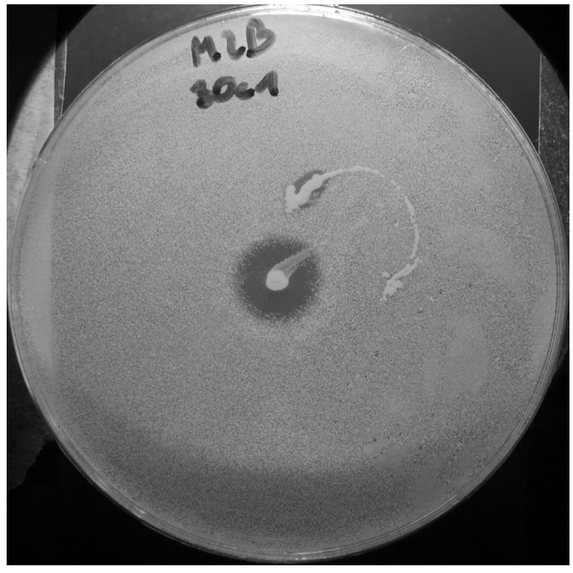

Fig. 1. Growth inhibition zone around the colony of producer strain M2B; an indicator strain – *S. aureus* 30C1.

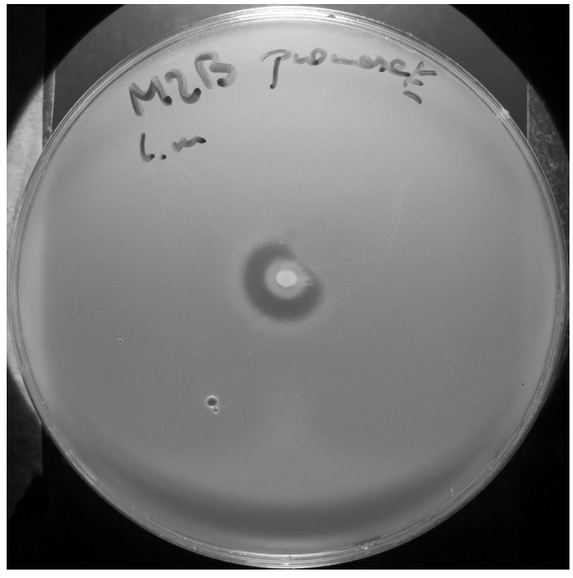

Fig. 2. Formation of "half moon zone" near the colony of producer strain M2B in the presence of proteolytic enzyme; an indicator strain – *L. monocytogenes*.

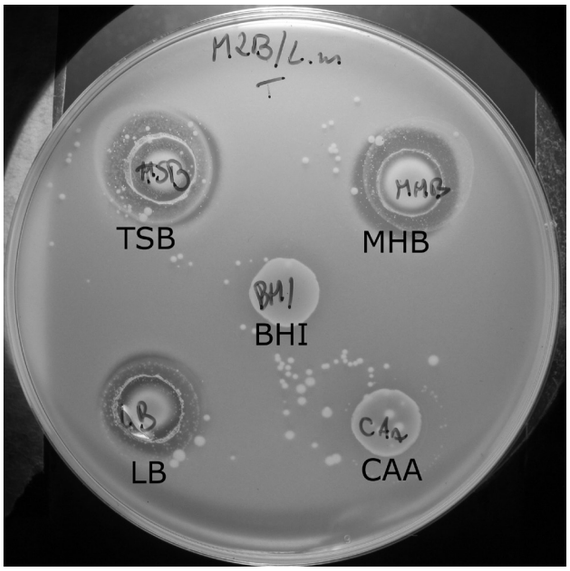

Fig. 3. Antimicrobial activity of the after-culture liquid media after growth of the producer strain.

the production efficiency in five different commercially available media, recommended for staphylococci culture, revealed that the optimal production of the putative AMP was observed in TSB medium. Slightly lower level of peptide production was found in MHB and LB media and no production was detected in CAA and BHI media (Fig. 2).

The maximum level of AMP production by *S. xylosus* M2B was observed after 24 h of incubation in TSB medium, and the optimal temperature was 37°C.

Table II
Activity of newly identified agent against strains from the Departments' collection.

No. of strain	Code number of the sensitive strain	Growth inhibition zone [mm]
1	7 (*S. aureus*)	12
2	11 (*S. aureus*)	20
3	28 (*S. aureus*)	15
4	52 (*S. aureus*)	22
5	68 (*S. aureus*)	25
6	77 (*S. aureus*)	18
7	92 (*S. aureus*)	17
8	99 (*S. aureus*)	25
9	115 (*S. aureus*)	15
10	83A (*S. epidermidis*)	15
11	8J (*S. xylosus*)	15
12	30C1 (*S. xylosus*)	15
13	247 (*S. xylosus*)	15
14	*L. monocytogenes*	15
15	*M. luteus*	15

The tests of thermostability revealed that the putative antimicrobial peptide did not lose its activity after 15 minutes of heating at 90°C. After 30 min of incubation in 90°C peptide lost its activity (inhibition zone was significantly smaller).

Discussion

Studies on the antimicrobial substances produced by staphylococci, have not been very extensive so far. However, there are a few peptides exhibiting antistaphylococcal activity produced by bacteria that belong to this genus, including both CNS – coagulase negative and CPS – coagulase positive staphylococci. The most promising examples of AMPs produced by CNS are the following bacteriocins: Pep5, epicidin 280, epilancin K7, epidermin, nukacin ISK-1, simulancin 3299, and the most extensively characterized bacteriocins produced by CPS are: staphylococcin C55, aureocin A70, aureocin A53 (Nascimento *et al.*, 2006; Varella Coelho *et al.*, 2007). The detailed characteristics of the best characterized staphylococcins has been presented in the review by Bastos and coworkers (Bastos *et al.*, 2009). The authors also emphasized that staphylococcins may be applied, solely or in a combination with other chemical agents. The use of combinations of antimicrobials is common in the clinical setting, and the most important advantages coming from such approach are: 1) expanding the spectrum of organisms that can be targeted, 2) prevention of the emergence of resistant organisms, 3) decreasing of toxicity by allowing lower doses of both agents (Kurlenda and Grinholc, 2012).

Our studies, results of which are presented in this work, are probably one of the very few examples of an extensive screening aimed at the identification of antimicrobial peptides produced by staphylococci isolated from humans (skin and soft tissue infections n = 48) or animals (strains isolated from bovine with mastitis n = 158). From the set of 206 strains, we identified six strains producing any antimicrobials (2.9%), of which, one was confirmed to be a putative peptide or protein with antimicrobial activity, efficiently produced in a liquid medium. Two out of three antimicrobial – producing strains identified in these studies did not produce the antimicrobial agents at the detectable level when grown in liquid media. Similar problems were recently observed by Braem and coworkers (2014), who identified *Staphylococcus chromogenes* L217 strain (isolated from teat apices of dairy cows) producing nukacin-like bacteriocin displaying a broad spectrum of antimicrobial activity. Unfortunately, the bacteriocin was only produced in significant quantities when the producer strain was grown on solid agar medium.

The molecular structure of the putative AMP produced by *S. xylosus* M2B is not known until now. This work is in progress in our laboratory, but preliminary results indicate that it could be a novel, previously undescribed compound. Its antimicrobial spectrum is interesting, since it is relatively narrow. Additionally, the good thermostability is advantageous, which could be especially important in the case of potential application of the identified antimicrobial peptide for the elimination of staphylococci from food products, but also for purification procedure, *e.g.* preliminary separation of the peptide from other components of the cell free after-culture supernatant by the heat treatment.

The produced peptide exhibits also strong activity against other important foodborne pathogenic bacteria *L. monocytogenes*. Thus, it could be also applied for protection of food products against these bacteria.

The recent studies carried out by Hewelt-Belka and coworkers (2016) revealed some important differences in the lipid composition of *S. aureus* clinical isolates. The observed, by the mentioned authors, differences in the lipid patterns between sensitive and resistant *S. aureus* strains suggest that antibiotic susceptibility may be associated with the lipid composition of bacterial cells. The lipids that were found to significantly differ between antibiotic-resistant and antibiotic-sensitive clinical isolates are involved in the biosynthesis of major *S. aureus* membrane lipids and lipoteichoic acid. The research of Powers and Hancock (2003) revealed that the lipid composition of the bacterial cell membrane is crucial for AMPs activity. Thus the observed in our studies differences in the susceptibility of *Staphylococcus* spp. strains tested to the agent produced by M2B can be caused by some differences in the lipid composition of their cell membranes.

In our studies, the prevalence of isolation of strains producing AMPs was found to be at a relatively low level – below 2% (three out of 206 isolates), and only one strain (prevalence below 0.5%) produced a peptide that is worthy of further studies. However, we do not have any doubts that staphylococci represents an interesting and promising source of uncharacterized antimicrobial peptides, which could be used for treatment or prophylaxis of infections caused by other bacteria that belong to the *Staphylococcus* spp. but also other important pathogenic microorganisms *e.g. L. monocytogenes*. Unfortunately, the research in this field is limited. Each year, numerous publications concerning the antibiotic resistance of the staphylococci isolated from both, human and animal (especially bovine mastitis) infections from different world regions are presented. In the case of some of these publications, large populations, counting even several hundreds of isolates, are investigated. Unfortunately, very few of these isolates are investigated for their potential production of antimicrobial agents. The methods used for screening the 'producing strains' are relatively easy and could be performed in most of microbiological laboratories. Thus, we encourage other research groups, which have access to the large number of human or animal isolates of staphylococci, for investigation of the production ability of potential antimicrobial peptides by these strains.

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Literature

Andersson D.I., D. Hughes and J.Z. Kubicek-Sutherland. 2016. Mechanisms and consequences of bacterial resistance to antimicrobial peptides. *Drug Resist. Updat.* 26: 43–57.

Barefoot S.F. and T.R. Klaenhammer. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* 45: 1808–1815.

Bastos M.C.F., H. Ceotto, M.L.V. Coelho and J.S. Nascimento. 2009. *Staphylococcal* antimicrobial peptides: relevant properties and potential biotechnological applications. *Curr. Pharm. Biotechnol.* 10: 38–61.

Braem G., B. Stijlemans, W. Van Haken, S. De Vliegher, L. De Vuyst and F. Leroy. 2014. Antibacterial activities of coagulase-negative staphylococci from bovine teat apex skin and their inhibitory effect on mastitis-related pathogens. *J. Appl. Microbiol.* 116: 1084–1093.

Brito M.A.V.P., G.A. Somkuti and J.A. Renye. 2011. Production of antilisterial bacteriocins by staphylococci isolated from bovine milk1. *J. Dairy Sci.* 94: 1194–1200.

Di Meo F., G. Fabre, K. Berka, T. Ossman, B. Chantemargue, M. Paloncýová, P. Marquet, M. Otyepka and P. Trouillas. 2016. In silico pharmacology: Drug membrane partitioning and crossing. *Pharmacol. Res.* 111: 471–486.

Fleming H.P., J.L. Etchells and R.N. Costilow. 1975. Microbial inhibition by an isolate of Pediococcus from Cucumber Brines 1. *Appl. Microbiol.* 30: 1040–1042.

Hewelt-Belka W., J. Nakonieczna, M. Belka, T. Bączek, J. Namieśnik and A. Kot-Wasik. 2016. Untargeted lipidomics reveals differences in the lipid pattern among clinical isolates of *Staphylococcus aureus* resistant and sensitive to antibiotics. *J. Proteome Res.* 4: 914–22.

Jakubczak A., P. Szweda, K. Łukaszewska and J. Kur. 2007. Molecular typing of *Staphylococcus aureus* isolated from cows with mastitis in the east of Poland on the basis of polymorphism of genes coding protein A and coagulase. *Pol. J. Vet. Sci.* 10: 199–205.

Joerger R.D. 2003. Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poultry Sci.* 82: 640–647.

Kosikowska P. and A. Lesner. 2016. Antimicrobial peptides (AMPs) as drug candidates: a patent review (2003–2015). *Expert Opin. Ther. Pat.* 26: 689–702.

Kot B., M. Piechota, M. Antos-Bielska, E. Zdunek, K.M. Wolska, T. Binek, J. Olszewska, P. Guliński and E.A. Trafny. 2012. Antimicrobial resistance and genotypes of staphylococci from bovine milk and the cowshed environment. *Pol. J. Vet. Sci.* 15: 741–749.

Kurlenda J. and M. Grinholc. 2012. Alternative therapies in *Staphylococcus aureus* diseases. *Acta Biochim. Pol.* 59: 171–184.
Lee H., J. Churey and R. Worobo. 2008. Antimicrobial activity of bacterial isolates from different floral sources of honey. *Int. J. Food Microbiol.* 126: 240–244.
Malanovic N. and K. Lohner. 2016. Antimicrobial peptides targeting Gram-positive bacteria. *Pharmaceuticals (Basel)*. 9(3). pii: E59. doi: 10.3390/ph9030059.
Nascimento J.S., H. Ceotto, S.B. Nascimento, M. Giambiagi-Demarval, K.N. Santos and M.C.F. Bastos. 2006. Bacteriocins as alternative agents for control of multiresistant staphylococcal strains. *Lett. Appl. Microbiol.* 42: 215–221.
Nes I.F. 2011. History, Current Knowledge, and Future Directions on Bacteriocin Research in Lactic Acid Bacteria. In: Drider D. and S. Rebuffat (eds). Prokaryotic Antimicrobial Peptides. Springer, New York, NY
Ołdak A. and D. Zielińska. 2017. Bacteriocins from lactic acid bacteria as an alternative to antibiotics. *Post. Hig. Med. Dosw.* 71: 328–338.
Powers J.-P.S. and R.E. Hancock. 2003. The relationship between peptide structure and antibacterial activity. *Peptides* 24: 1681–1691.
Rashid R., M. Veleba and K.A. Kline. 2016. Focal targeting of the bacterial envelope by antimicrobial peptides. *Front. Cell Dev. Biol.* 4:55. doi: 10.3389/fcell.2016.00055.
Schillinger U. and F.K. Lücke. 1989. Antibacterial activity of Lactobacillus sake isolated from meat. *Appl. Environ. Microbiol.* 55: 1901–1906.
Sjölund M. and G. Kahlmeter. 2008. Staphylococci in primary skin and soft tissue infections in a Swedish county. *Scand. J. Inf. Dis.* 40: 894–898.
Szweda P., M. Schielmann, A. Frankowska, B. Kot and M. Zalewska. 2014. Antibiotic resistance in *Staphylococcus aureus* strains isolated from cows with mastitis in eastern Poland and analysis of susceptibility of resistant strains to alternative nonantibiotic agents: lysostaphin, nisin and polymyxin B. *J. Vet. Med. Sci.* 76: 355–362.
Varella Coelho M.L., J.D. Santos Nascimento, P.C. Fagundes, D.J. Madureira, S.S Oliveira, M.A. Vasconcelos de Paiva Brito and C. Freire Bastos Mdo. 2007. Activity of staphylococcal bacteriocins against *Staphylococcus aureus* and *Streptococcus agalactiae* involved in bovine mastitis. *Res. Microbiol.* 158: 625–630.
Weisburg W.G., S.M. Barns, D.A. Pelletier and D.J. Lane. 1991. 16S ribosomal DNA ampigication for phylogenetic study. *J. Bacteriol.* 173: 697–703.
Yeaman, M.R. and N.Y. Yount. 2003. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* 55: 27–55.

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ORIGINAL PAPER

Genetic Analysis Method for *Staphylococcus chromogenes* Associated with Goat Mastitis

ROCÍO A. RUIZ-ROMERO[1], ROBERTO A. CERVANTES-OLIVARES[2], ANDRÉS E. DUCOING-WATTY[1], DANIEL MARTÍNEZ-GÓMEZ[3]*, EFRÉN DÍAZ-APARICIO[4] and ESTELA T. MÉNDEZ-OLVERA[5]

[1] Departamento de Medicina y Zootecnia de Rumiantes, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, México
[2] Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, México
[3] Laboratorio de Microbiología Agropecuaria, Departamento de Producción Agrícola y Animal, Universidad Autónoma Metropolitana, México
[4] Centro Nacional de Investigación Disciplinaria- Microbiología Animal, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, México
[5] Laboratorio de Biología Molecular, Departamento de Producción Agrícola y Animal, Universidad Autónoma Metropolitana, México

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Abstract

Mastitis in goats is mainly caused by coagulase-negative *Staphylococcus* (CNS). The identification methods for this group are based on evaluating the expression of phenotypic characteristics such as the ability to metabolize various substrates; however, this is disadvantageous as these methods are dependent on gene expression. In recent years, genotyping methods such as the Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA) and gene identification have been useful for epidemiological study of several bacterial species. To develop a genotyping method, the genome sequence of *Staphylococcus chromogenes* MU970 was analysed. The analysis showed nine virulence genes described in *Staphylococcus aureus*. The MLVA was developed using four loci identified in the genome of *S. chromogenes* MU970. This genotyping method was examined in 23 strains of CNS isolated from goat mastitis. The rate of discrimination for MLVA was 0.8893, and the highest rates of discrimination per the index of Simpson and Hunter-Gaston were 0.926 and 0.968 for the locus 346_06, respectively. The virulence genes were present in all strains of *S. chromogenes* but not in other CNS. The genotyping method presented in this paper is a viable and easy method for typifying CNS isolates from mastitis cases in different regions and is an ideal mean of tracking this disease.

Key words: genotyping, mastitis in goats, MLVA, *Staphylococcus chromogenes,* VNTR

Introduction

Goat mastitis is primarily caused by CNS. CNS are classified into 47 species and 23 subspecies (Becker *et al.*, 2014), and in mastitis cases, more than 10 CNS species have been isolated (Taponen and Pyörälä, 2009; Zadocks and Watts, 2009). The CNS represent a heterogeneous group within the *Staphylococcus* genus, and the identification methods for this group evaluate the expression of genetically encoded characteristics, such as enzyme production (Stepanovick *et al.*, 2006; Becker *et al.*, 2014; Vanderhaeghem *et al.*, 2015). One disadvantage of the phenotypic identification method is the expression variability of phenotypic traits between isolates of the same species, and because of this, genotypic identification methods have been developed (Monir *et al.*, 2007). These methods are based on the genetic material analysis of the organism; therefore, they are independent of changes in the gene expression pattern. Molecular methods represent a more stable and reproducible alternative and provide useful information about the genetic connections between isolates from different sources, allowing epidemiological monitoring of disease outbreaks (Hollender *et al.*, 2013; Wang *et al.*, 2016). Genotyping bacteria by typing their loci containing a variable number of tandem repeats (VNTR) may become the gold standard for many pathogens (Ramisee *et al.*, 2004; Vergnaud and

* Corresponding author: D. Martínez-Gómez, Laboratorio de Microbiología Agropecuaria, Departamento de Producción Agrícola y Animal, Universidad Autónoma Metropolitana Unidad Xochimilco, Calzada del hueso, Villa Quietud, Delegación Coyoacán, México; **e-mail: dmartinez@correo.xoc.uam.mx**

Pourcel, 2009). MLVA is a DNA-based molecular typing method frequently used in the study of prokaryotes. It records size polymorphisms in several VNTR loci amplified by stringent PCR protocols (Le Fleche *et al.*, 2001). MLVA is useful in epidemiology because it replaces older, slower and dangerous methodologies (phenotypic identification methods require handling live pathogenic bacteria) for typing microorganisms. VNTRs are a powerful tool for determining evolutionary relationships and population genetics of bacteria (Hardy *et al.*, 2004). The development of genome sequencing has shown that VNTR sequences are present in many bacterial species and that polymorphism exists in most of them. These repetitions have become a source for locating markers to identify pathogenic bacteria (Vergnaud and Pourcel, 2009). In *S. aureus*, MLVA is useful for strain typing. An MLVA analysis of 130 strains isolated from raw-milk dairy products (122 isolates) and human samples (eight isolates) revealed marked genomic variability among the samples. In this study, the MLVA technique correctly assigned isolates from outbreaks and discriminated isolates that were not from outbreaks (Ikawaty *et al.*, 2008). In this study, we developed the MLVA for *S. chromogenes* using the *S. chromogenes* MU970 genome sequence (NCBI Reference Sequence: NZ_JMJF00000000.1). This genotyping method included nine virulence genes previously described in other *Staphylococcus* spp.

Experimental

Materials and Methods

Bacterial isolates. Fifty-three *Staphylococcus* strains were isolated from the milk of both healthy goats and goats with subclinical mastitis from farms in Queretaro and Guanajuato, Mexico. To isolate the strains, the milk was plated on trypticase soy agar and incubated at 37°C for 24 hours. The microorganisms were Gram-stained and catalase tested to identify the *Staphylococcus* isolates. Coagulase tests were performed to identify coagulase-negative *Staphylococcus*, using the API Staph® (V4.1) Biomerieux laboratory system per the manufacturer's instructions. A chemotherapeutic susceptibility test was conducted on all isolates identified as *S. chromogenes* using the Kirby-Bauer technique (Bernal and Guzman 1984) with antimicrobial susceptibility disks (polymyxin B, ampicillin, tobramycin, gentamicin and tetracyclines) (BD Becton-Dickinson and Company).

DNA extraction and polymerase chain reaction (PCR). DNA extraction from the 53 bacterial isolates was made as per the protocol described by Cremonesi *et al.* (2006). DNA concentration was measured by spectrophotometry (BIORAD SmartSpec-Plus Spectrophotometer). To identify the *Staphylococcus* genus, primers designed by Mason *et al.* (2001) were used. To identify the isolates within the CNS group, we used a pair of primers corresponding to the *coa* gene of *S. aureus*. This primer amplifies a 1200-bp fragment from these genes (Ruiz *et al.*, 2013). DNA from *S. chromogenes* ATCC® 43764 TM and *S. aureus* ATCC® 29737 TM were used to validate the PCR test (PCR Master Mix, Fermentas). The thermocycler conditions were specific for each primer. The PCR products were analysed by electrophoresis on 1% agarose gel using TAE 1X as the running solution. To identify the virulence genes in *S. chromogenes*, nine pairs of primers were designed to amplify different proteins defined as virulence factors in *S. aureus*. Primer design was performed by obtaining the amino acid sequence of the aim genes, using the BLAST database (Basic Local Alignment Search Tool) from the NCBI (National Center of Biotechnology Information; http://blast.ncbi.nlm.nih.gov/Blast.cgi) for verifying the sequence homology. We then located the amino acid sequence within the genome, and the primers were designed using the DNAman program (Lynnon Corporate) version 7.02 for Windows (Table I).

MLVA. The *S. chromogenes* MU970 strain genome was analysed to locate the tandem repetitions using the Tandem Repeats Finder Program (Benson, 1999). The primers of the loci flanking regions from the tandem repetitions were designed using the DNAman v. 7.02 program. Six loci of 15- to 96-bp were selected from the tandem repetitions, with the number of copies ranging from 7 to 23, to observe the amplifications on an agarose gel. Tandem repetitions were named according to the genome where they were found and the size of the expected product in the *S. chromogenes* MU970 strain (Table II). MLVA analysis was performed with all *S. chromogenes* isolates. The PCR products of the repeated sequences were analysed with the fragment QIAxcel DNA High Resolution Kit (QIAGEN México S. DE R.L. DE C.V. CP 01090, México City. Catalogue number 929002) analyser, following the manufacturer's instructions. The size of each VNTR was determined using a molecular weight marker of 100- to 3000-bp.

Data Analysis. The size of each amplicon was determined using the molecular weight marker of 3000-bp, and the repetition number of each allele was derived from the size of the obtained amplicon. For each VNTR locus, we calculated the diversity indexes of Simpson and Hunter-Gaston (1988) with confidence intervals of 95%, using the VNTR Diversity and Confidences Extraction Software program (V-DICE), from the Health Protection Agency website (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl). The MLVA discrimination power was calculated using the Discriminator Power Calculator program (http://insilico.

Table I
Primers used to identify *S. chromogenes.*

Primers	Sequence (5'-3')	Conditions	Size [bp]
16S rRNA	F'- CCTATAAGACTGGGATAACTTCGGG R'- CTTTGAGTTTCAACCTTGCGGTCG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	791
coa	F'- CAAAGCAGATGCGATAG R'- CCTGTACCAGCATCTCTAT	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	1200
Aureolysin	F'- GCATTAAACGAAASCTTYTCWG R'- GTATGCAGCTTTATTTGGWATACC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	247
Hemolysin	F'- ATGAATATTGGAATAACTTTAGTCAG R'- TTAGGAAGCATACAATTGATGT	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	600
Extracelular protein	F'- ACGTGAGCAATATATGAACGC R'- TTATAAATACCTGTTAATGCGCC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	133
Zin Metaloprotease	F'- ATTTGCAATCGGGATGG R'- ATGTTGATCATCTAAAATAATATGCG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	250
Surface protein SasH	F'- GGCWAAAGCRATGAATGC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m,	201
	R'- ACCGATAATKCCRTAACG	72°C/1 m/ 1 cycle 72°C/5 m.	
TRAP (Transduction signal protein)	F'-AACTCTATTCACTTATGGAACATATGG R'- CTGTTCAACATTTTGCTGTTG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	529
B antigen	F'- CAAAAACACTATTAGCGACTGG R'- CTTTTATGACGATGGAGTTTCC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	488
Virulence B factor	F'- ATGTCTTTTAAAGAAAATGAAATCG R'- TATGCATGATTTTCACTGTGC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	905
FemA protein	F'- GTGTGCTTRTACCWYTAGC R'- CCAGCATAATAAACWASTTC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	250

Table II
Primers used to amplify the VNTR.

Primer	Sequence (5'-3')	Conditions	Size [bp]
266_07	F'- ATTCTGGATTTTGCACAGC R'- ATCTGCTAAAATGACAGAATTACAAC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	266
346_06	F'- CAAAACAACGATATCTGTATCTGA R'- TGTCGGTTTATTAGGTTGAGAAG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	346
360_06	F'- CAAGTGCATAACCGTTATTCC R'- TGTCTGATGTCGGTTTATTAGG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	360
854_08	F'- GAAGCACTTACACTTTCGGAG R'- GACTTCACTAAGTGAGTCAACAAGTAC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	854
638_07	F'- TGAGGTCTCCGCTGTAGG R'- GCAGACGTCCCTGTTGAG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	638
613_12	F'- AGTAGCTAACCATTTTGTTAATTGC R'- GTTAAAGAAAATTCTTCACAGTCG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	613

ehu.es/mini_tools/discriminatory_power/index.php). The 23 *S. chromogenes* isolates were grouped by their phenotypic characteristics through a cluster analysis, using the Euclidean distance and the Ward minimum variance method with the DELL STATISTICA (Data Analysis Software System) program. The cluster analysis of the *S. chromogenes* genogroups was performed with the BioNumerics software version 7.6.1 (Applied Maths, St-Martens-Latern, Belgium) using the Pearson correlation coefficient and the pair grouping method with an unweighted arithmetic mean (UPGMA).

Results

Bacterial Identification. The 53 bacterial isolates were Gram-positive cocci and were grouped in clusters of catalase positive and coagulase negative using a macroscopic morphology characteristic of the genus. From the results of the API system Staph®, 23 isolates corresponded to *S. chromogenes*, 18 to *Staphylococcus simulans*, nine to *Staphylococcus xylosus*, two to *S. sciuri*, and one to *Staphylococcus warneri*. These results, as well as the results of the chemotherapeutic susceptibility

test for the isolates identified as *S. chromogenes*, are shown in Table III.

Molecular identification. PCR was standardized for the detection of the 16s *Staphylococcus* region and for the *coa* gene from *S. aureus* to confirm the expected size of each product using DNA from the ATCC strains at concentrations of 100 ng/µl. The 53 isolates were positive for the 16s gene and negative for the *coa* gene. The *S. chromogenes* isolates were positive for all virulence genes, except for the gene encoding the *virulence B factor*, in which only 95.65% of the isolates were positive. All *S. simulans* isolates were positive for the genes encoding the extracellular protein, zinc metalloprotease, SasH and FemA, and 27.78% were positive for the aureolysin gene, 16.67% for the *TRAP* gene and 11.11% for haemolysin and B antigen. In addition, all of these were negative for the virulence B factor. All *S. xylosus* isolates were positive for the extracellular protein genes, zinc metalloprotease and FemA. Of these, 66.67% were positive for SasH, 44.44% for aureolysin, and 33.33% for SasH and virulence B factor; however, none of these isolates were positive for the B antigen. All *S. sciuri* isolates were positive for all virulence genes except haemolysin and the B antigen, for which none of the isolates were positive. Finally, the *S. warneri* isolate was positive for the extracellular protein, zinc metalloprotease and the virulence B factor and was negative for the rest of the virulence genes (Table IV).

Phenotypic characteristics. To determine the variability of the 23 *S. chromogenes* isolates from the different biochemical tests and the chemotherapy susceptibility test, a dendrogram was constructed showing three clusters of the 23 *S. chromogenes* isolates. The first cluster contains six isolates, divided into two subgroups. In the first subgroup, there are two isolates from farm number 2, which has an intensive production system, and four isolates from farm number 1, which has an extensive production system. This group is characterized for being maltose-negative, having a variable N-acetyl-glucose response, and being sensitive to the five antibiotics that were used. The second cluster has six isolates from farm number 3, which are divided into two subgroups. In this cluster, the isolates are maltose-positive, N-acetyl-glucose-negative, and polymyxin B and ampicillin-resistant. The third cluster has one subgroup with two isolates from farm number 1, two isolates from farm number 2 and five isolates from farm number 3, which has an intensive production system. This cluster is characterized as being maltose-positive, N-acetyl-glucosamine-positive and polymyxin B and ampicillin-resistant. In the principal component analysis, the *S. chromogenes* isolates are further grouped according to cluster analysis (Fig. 1).

MLVA. The *S. chromogenes* isolates were used to perform the MLVA, and 63 VNTR loci were identified in the genome of *S. chromogenes* MU970. Six were used for the *S. chromogenes* isolates, and the amplification of four loci was achieved in the 23 isolates (Table V). From the VNTR, the 23 isolates were grouped using the Unweighted Pair Group Method with Arithmetic

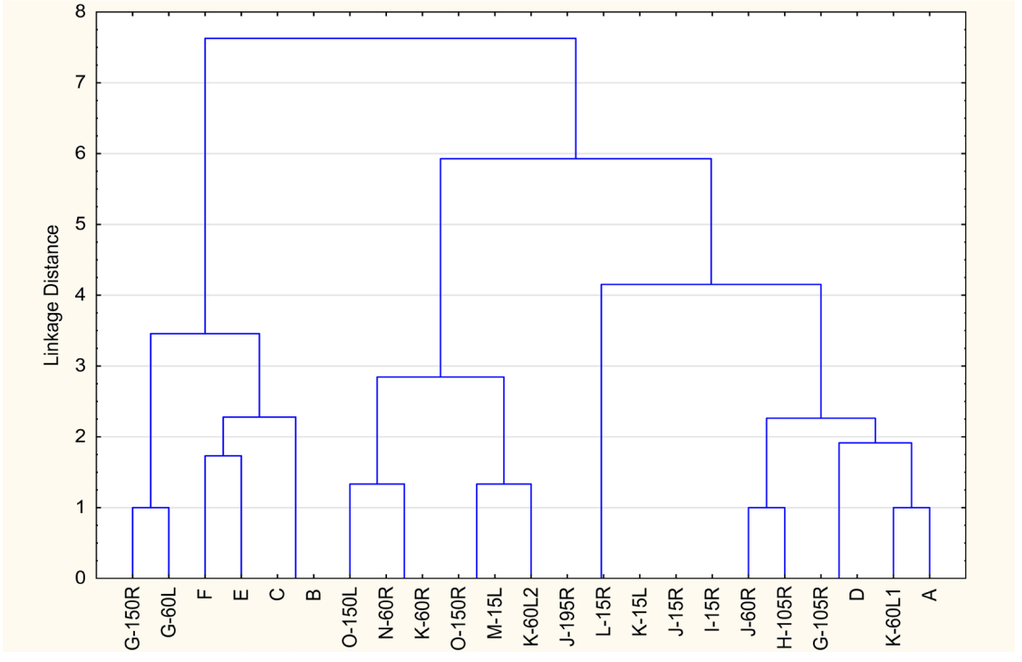

Fig. 1. Cluster analysis according to the phenotypic characteristics and chemotherapeutic susceptibility of 23 *S. chromogenes* strains according to Ward's minimum variance method.

Table III
Biochemical tests results with Api Staph System and antibiograms.

Strain	Origin	Goat id	Farm	Production system	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	Guanajuato	A	1	Extensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	R	S	S	S	S
2	Guanajuato	B	1	Extensive	+	+	+	–	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	S	S	S	S	S
3	Guanajuato	C	1	Extensive	+	+	+	–	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	S	S	S	S	S
4	Guanajuato	D	1	Extensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	S	R	S	S	S
5	Guanajuato	E	1	Extensive	+	+	+	–	–	+	+	–	–	+	+	–	–	–	+	–	–	+	+	S	S	S	S	S
6	Guanajuato	F	1	Extensive	+	+	+	–	+	+	–	–	–	+	+	–	–	–	+	–	–	+	+	S	R	S	S	S
7	Querétaro	G-105R	2	Intensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	S	R	S	S	S
8	Querétaro	H-105R	2	Intensive	+	–	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	S	R	S	S	S
9	Querétaro	G-60L	2	Intensive	+	+	–	–	+	+	+	–	–	+	+	–	–	–	+	–	+	+	–	S	S	S	S	S
10	Querétaro	G-150R	2	Intensive	+	+	–	–	+	+	+	–	–	+	+	–	+	–	+	–	+	+	–	S	S	S	S	S
11	Querétaro	I-15R	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	R	R	S	S	S
12	Querétaro	J-15R	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	R	R	S	S	S
13	Querétaro	K-15L	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	R	R	S	S	S
14	Querétaro	J-60R	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	S	R	S	S	S
15	Querétaro	K-15L1	3	Intensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	R	R	S	S	S
16	Querétaro	K-15L2	3	Intensive	+	+	–	+	+	+	–	–	–	+	+	–	–	–	+	–	–	+	+	R	R	S	S	S
17	Querétaro	K-15R3	3	Intensive	+	+	+	+	+	+	–	–	–	+	+	+	–	–	+	–	–	+	+	S	R	S	S	S
18	Querétaro	L-15R	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	I	R	S	S	S
19	Querétaro	J-195R	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	I	R	S	S	S
20	Querétaro	M-15L	3	Semiintensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	–	+	+	R	R	S	S	S
21	Querétaro	N-60R	3	Semiintensive	+	+	+	+	+	+	–	–	–	+	+	+	–	–	+	–	–	+	+	S	R	S	S	S
22	Querétaro	O-150L	3	Semiintensive	+	+	+	+	+	+	–	–	–	+	+	+	–	–	+	–	–	+	+	I	R	S	S	S
23	Querétaro	O-150R	3	Semiintensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	–	+	+	R	R	S	S	S

The physiological tests used with *S. chromogenes* were:
1. Glucose; 2. Fructose; 3. Manose; 4. Maltose; 5. Lactose; 6. Trehalose; 7. Manitol; 8. Xilitol; 9. Mellobiose; 10. Nitrates; 11. Alkaline Phosphatase; 12. Voges-Proskauer; 13. Rafifnose; 14. Xylose; 15. Saccharose; 16. Metil-Gluco-Pyranosidase; 17. N-Acetyl-Glucose; 18. Arginin Dihydrolase; 19. Urea; 20. Polymyxin B; 21. Ampicillin; 22. Gentamicine; 23. Tobramycine and 24. Tetracycline.

Table IV
The genes identified in the isolated CNS.

The gen coding for	*S. chromogenes*		*S. simulans*		*S. xylosus*		*S. sciuri*		*S. warneri*	
	+/total	%	+/total	%	+/total	%	+/total	%	+/total	%
Aureolysin	23/23	100	5/18	27.78	4/9	44.44	2/2	100	0/1	0
Hemolysin	23/23	100	2/18	11.11	0/9	0	0/2	0	0/1	0
Extracellular protein	23/23	100	18/18	100	9/9	100	2/2	100	1/1	100
Zin metaloprotease	23/23	100	18/18	100	9/9	100	2/2	100	1/1	100
Surface protein SasH	23/23	100	18/18	100	6/9	66.67	2/2	100	0/1	0
TRAP	23/23	100	3/18	16.67	3/9	33.33	2/2	100	0/1	0
B Antigen	23/23	100	2/18	11.11	0/9	0	0/2	0	0/1	0
Virulence B factor	22/23	95.65	0/18	0	3/9	33.33	2/2	100	1/1	100
FemA protein	23/23	100	18/18	100	9/9	100	2/2	100	0/1	0

Table V
Number of VNTR detected by capillary electrophoresis.

Strain	Origin	Farm	Production system	266_07	VNTR	346_06	VNTR	360_06	VNTR	854_08	VNTR
1	Guanajuato	1	Extensive	955	27	1092	73	1187	40	1101	11
2	Guanajuato	1	Extensive	955	27	1166	78	1156	39	301	3
3	Guanajuato	1	Extensive	963	27	1153	77	1183	39	1101	11
4	Guanajuato	1	Extensive	953	26	1161	77	1181	39	1077	11
5	Guanajuato	1	Extensive	952	26	1159	77	1181	39	1210	13
6	Guanajuato	1	Extensive	936	26	1164	78	1175	39	1201	13
7	Querétaro	2	Intensive	453	13	1181	79	1175	39	329	3
8	Querétaro	2	Intensive	453	13	1177	78	1177	39	329	3
9	Querétaro	2	Intensive	1075	30	1176	78	1176	39	1221	13
10	Querétaro	2	Intensive	1080	30	1178	79	1182	39	1229	13
11	Querétaro	3	Intensive	384	11	1155	77	1176	39	329	3
12	Querétaro	3	Intensive	383	11	1157	77	1178	39	329	3
13	Querétaro	3	Intensive	385	11	1156	77	1172	39	329	3
14	Querétaro	3	Intensive	382	11	1164	78	1181	39	204	2
15	Querétaro	3	Intensive	382	11	1150	77	1181	39	329	3
16	Querétaro	3	Intensive	382	11	1155	77	1183	39	330	3
17	Querétaro	3	Intensive	385	11	1178	79	1181	39	329	3
18	Querétaro	3	Intensive	989	27	1158	77	1181	39	329	3
19	Querétaro	3	Intensive	384	11	1156	77	1178	39	329	3
20	Querétaro	3	Semi-intensive	383	11	1168	78	1186	40	328	3
21	Querétaro	3	Semi-intensive	386	11	1163	78	1186	40	205	2
22	Querétaro	3	Semi-intensive	872	24	1192	79	1185	40	329	3
23	Querétaro	3	Semi-intensive	0	0	1187	79	1186	40	329	3

Mean (UPGMA) and the Pearson correlation coefficient. An 85.7% similarity was obtained for the 23 isolates, which were then grouped in ten genogroups and divided into two larger groups. Per the MLVA results, the six Guanajuato isolates are grouped with four isolates from Queretaro with a 93.2% similarity, while the 13 remaining isolates from Queretaro are grouped with a 90.4% similarity (Fig. 2). The discrimination ability of the MLVA was determined using the discrimination index (*D*) for the 23 isolates, which showed discrimination level with a D value of 0.8893. The discrimination power of each VNTR was estimated from the number of alleles detected and their diversity. The highest diversity rates using the Simpson and Hunter-Gaston index were 0.926 and 0.968, respectively, for the 346_06 locus, whereas the lowest diversity indexes were from the 854_08 locus with rates of 0.654 and 0.684, respectively (Table VI).

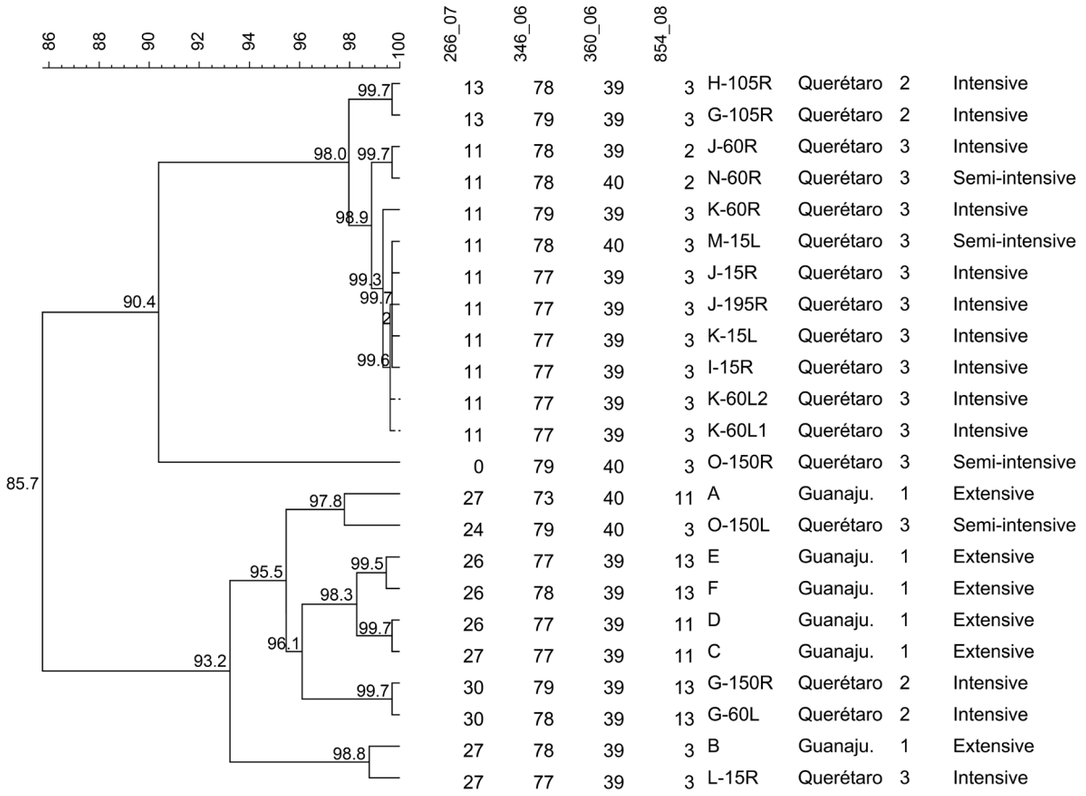

Fig. 2. Cluster analysis according to the VNTR of 23 strains of *S. chromogenes* using Pearson's correlation coefficient and the UPGMA algorithm.

Discussion

The *Staphylococcus* genus is commonly isolated from mastitis cases in ruminants. Subclinical mastitis in goats is primarily caused by coagulase-negative *Staphylococcus*, which is considered a minor and opportunistic pathogen (Bergonier *et al.*, 2003; Vliegher *et al.,* 2004; Schukken *et al.,* 2009; Taponen and Pyorala, 2009). This group can cause mastitis due to its many virulence factors, both in human and animal isolates. Some of these virulence genes are shared with *S. aureus*, such as haemolysins, leucocidins, toxins, biofilm formation and adhesins (Pyörälä and Taponen, 2009; Taponen and Pyorala, 2009; Vergnaud and Pourcel, 2009; Park *et al.*, 2011; Supré *et al.*, 2011; Vanderhaeghem *et al.*, 2015). In this study, some of the virulence factors that are shared with *S. aureus* corresponded to aureolysin, extracellular metalloproteinase, and SasH protein. Although CNS species are less virulent than *S. aureus*, the different virulence factors in this group could influence their clinical features and the persistence of intramammary infections, which should be regarded as pathogenic and are not part of the normal microbiota (Haveri *et al.*, 2007; Schukken *et al.*, 2009; Taponen and Pyorala, 2009). We identified the isolates of *S. chromogenes* with *S. chromogenes* MU970 genes, which is presently the only reported sequence and was isolated from a bovine mastitis case in the United States.

Table VI
Diversity Index (Simpson and Hunter-Gaston) and confidence intervals for each VNTR loci for *S. chromogenes.*

Locus	Size [bp]	Simpson index	Confidence intervals 95%	Hunter-gaston index	Confidence intervals 95%
266_07	36	0.87	0.803–0.936	0.909	0.843–0.975
346_06	15	0.926	0.896–0.957	0.968	0.938–0.999
360_06	30	0.756	0.650–0.862	0.791	0.684–0.897
854_08	96	0.654	0.449–0.859	0.684	0.479–0.888

This type of CNS is the primary cause of subclinical mastitis in goats worldwide (Ruiz *et al.*, 2013). In this work, we detected adhesins, antigens associated with the cell wall and haemolysins, which are shared with other species of CNS. From these results and those in the literature, the presentation of the mammary gland disease and the resultant damage is influenced by the virulence factors that are present in the bacteria. Because there are no studies that focus on a single species in this group, it is important to know the virulence factors of CNS mastitis in ruminants to develop control measures and disease prevention (Schukken *et al.*, 2009; Sampaio *et al.*, 2015; Vanderhaeghem *et al.*, 2015). From the identification of these virulence genes in *S. chromogenes*, it is clear that the mastitis caused by this bacterium is significant; therefore, we developed the first specific MLVA for *S. chromogenes* isolates. Other studies used different genotyping methods rarely focus on a particular species; for example, authors have standardized genotyping methods for *S. epidermidis* isolates from bovine milk and human skin using PFGE, where five patterns were obtained. One of these skin isolates showed a pattern common to animal milk, indicating that it may be zoonotic (transmitted from humans to animals) (Thordberg *et al.*, 2006). For *S. chromogenes*, Shimizu *et al*. (1997) reported genotyping by PFGE of 138 *Staphylococcus hyicus* isolates from pigs, chickens, cows and goats, and 21 isolates of *S. chromogenes* obtained from pigs and dairy cows with mastitis. The patterns obtained for *S. hyicus* were different according to the animal's country of origin, whereas the *S. chromogenes* patterns were more conservative; however, the authors managed to differentiate them from the *S. hyicus* isolates (Shimizu *et al*., 1997). The MLVA presented here, could be used for molecular epidemiology studies on *S. chromogenes*, as well as other CNS species, since other species, identified in this work, were also isolated from subclinical mastitis cases in goats. Therefore, this analysis would be useful to examine CNS isolates from both animals and humans, in different geographic locations, in order to compare the genetic diversity within this group and standardize genotyping techniques. It is worth noting the importance of genotyping methods, as per the results of our cluster analysis that revealed three large clusters. Two of them comprised isolates from Queretaro and Guanajuato, whereas the third cluster contained only isolates from Queretaro. The main disadvantage of these phenotypic identification methods is that they determine biochemical and/or physiological traits. These phenotypic methods represent the first tool for comparing microorganisms and are an important tool for characterizing many pathogens; however, phenotypic traits are susceptible to environmental influence, which can cause variation in gene expression. Therefore, these results would be less stable, less reproducible and less discriminatory, making it difficult to determine whether the isolates have the same genetic pattern (Vilchez and Alonso, 2009). In this study, the MLVA classified the six isolates from Guanajuato within a cluster with four isolates from Queretaro, whereas the rest of the isolates from the two farms of Queretaro are grouped in a second cluster. In addition, isolates from the same goat were obtained on different lactation days, which indicates that the bacteria can survive for long periods of time in the mammary gland. In addition, isolates from different animals on the same farm belonged to the same genogroup, indicating that the isolates were from the same strain, and transmission was likely due to improper hygiene and/or management practices during milking. Furthermore, these results indicate that the genogroups of *S. chromogenes* differ based on geographical location since the genotypic identification methods developed in this work can be used to classify closely related or divergent microorganisms. Thus, the knowledge derived from epidemiological studies can determine the natural history of a disease, its aetiology, occurrence frequency, distribution, pathways and spreading patterns, reservoirs and factors that increase the risk of contracting the disease (Vilchez and Alonso, 2009). The discrimination index of the *S. chromogenes* MLVA was 0.8893, indicating that this method is capable of distinguishing between different isolates, because a D value of 1 indicates that the genotyping method is able to distinguish each member of the population from any other member of the same population, while an index of 0 indicates that all members of the population are identical, and a 0.50 index indicates that if an isolate is chosen randomly, there is a 50% chance that the next isolate chosen will be indistinguishable from the first (Hunter and Gaston, 1988; Zaluga *et al*., 2013). The highest discrimination rate pertained to the 346_06 locus, representing a viable option for genotyping the *S. chromogenes* isolates, since this locus present more variability. The MLVA has several advantages, such as the use of common and inexpensive laboratory equipment. One of the most important features of the MLVA is that analysis of a limited number of loci can provide information about the diversity that exists within the species, which is crucial for typifying different bacteria (Vergnaud and Pourcel, 2009). Tandem repetitions are useful as molecular markers because they represent one of the most diverse genomic loci in bacterial populations. They are made of smaller sequences known as repetition units or motifs, which are repeated in tandem and vary in their number of repeated copies (Van Belkum *et al.*, 1998; Vogler *et al.*, 2006,). These repeated copies result from insertion mutations and/or deletions, so they can gain or lose several of these repetition units and create many alleles. It has been pro-

posed that these mutations occur primarily due to a mismatch by strain landslides, but recombination events also occur (Taylor *et al.*, 2000; Vogler *et al.*, 2006). Most VNTR do not have any phenotypic effects and generate neutral genetic variations, so they have not been associated with biological effects (Vogler *et al.*, 2006). The speed of these repetition sequences remains unclear, so studies to determine the mutation rate would facilitate molecular epidemiologic investigations. In conclusion, the MLVA presented in this paper is an easy and viable method of typying CNS isolates from mastitis cases from different regions and is an ideal option for tracking this disease.

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Literature

Becker K., C. Heilmann and G. Peters. 2014. Coagulase-negative staphylococci. *Clin. Microbiol. Rev*. 27: 870–925.

Benson G. 1999. Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Res.* 27: 573–580.

Bergonier D., R. De Cremoux, R. Rupp, G. Lagriffoul and X. Berthelot. 2003. Mastitis of dairy small ruminants. *Vet. Res*. 34: 689–716.

Bernal M. and M. Guzmán. 1984. The Disc Antibiogram. Standardization of the Kirby-Bauer technique (El Antibiograma de Discos. Normalización de la técnica de Kirby-Bauer). *Biomédica* 4: 112–121.

Cremonesi P., B. Castiglioni, G. Malferrari, I. Biunno, C. Vimercati, P. Moroni, S. Morandi and M. Luzzana. 2006. Improved method for rapid extraction of mastitis pathogens directly from milk. *J. Dairy. Sci.* 89:168–169.

Fry P., M. Calcutt, M. Foecking, H. Hsieh, D. Suntrup, J. Perry, G. Stewart and J. **Middleton.** 2014. Draft genome sequence of a *Staphylococcus chromogenes* strain MU 970, isolated from a case of chronic bovine mastitis. *Genome Announc.* 2:e00835-14. doi:10.1128/genomeA.00835-14.

Hardy J., D. Ussery, B. Oppenheim and P. Hawkey. 2004. Distribution and characterization of staphylococcal interspersed repeat units (SIRUs) and potential use for strain differentiation. *Microbiol.* 150: 4045–4052.

Haveri M., A. Roslöf, L. Rantala and S. Pyörälä. 2007. Virulence genes of bovine *Staphylococcus aureus* from persistent and nonpersistent intramammary infections with different clinical characteristics. *J. Appl. Microbiol.* 103: 993–1000.

Hollender D., S. Conde, E. Salustio and L. Samartino. 2013. Detección de un complejo clonal con el genotipo de *Brucella abortus* biovariedad 2 como fundador de aislamientos de *B. abortus* en argentina. *Rev. Argent. Microbiol.* 45: 229–239.

Hunter P. and M. Gaston. 1988. Numerical index of the discriminatory ability of typing systems; an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26: 2465–2466.

Ikawaty R., J. Rob, T. Adrienne and J. Verhoef. 2008. Novel multiple-locus variable-number tandem-repeat analysis method for rapid molecular typing of human Staphylococcus aureus. *J. Clin. Microbiol.* 46: 3147–3151.

Le Fleche P., Y. Hauck, L. Onteniente, A. Prieur, F. Denoeud, V. Ramisse, P. Sylvestre, G. Benson, F. Ramisse and G. Vergnaud. 2001. Tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis. BMC Microbiology*. doi: 10.1186/1471-2180-1-2.

Mason W., J. Blevins, K. Beenken, N. Wibowo, N. Ojha and M. Smelter. 2001. Multiplex PCR protocol for the diagnosis of staphylococcal infection. *J. Clin. Microbiol*. 39: 3332–3338.

Monir S., H. Ihara, M. Noda, S. Xiao, P. Hong, K. Ohkusu and Y. Kawamura. 2007. DNAJ gene sequence-based assay for species identification and phylogenetic grouping in the genus *Staphylococcus. Int. J. Syst. Evol. Microbiol*. 57: 25–30.

Park Y., L. Fox, K. Seo. M. McGuire, Y. Park. F. Rurangirwa, W. Sischo and G. Bohach. 2011. Detection of Classical and Newly Described Staphylococcal Superantigen Genes in Coagulase-Negative Staphylococci Isolated from Bovine Intramammary Infections. *Vet. Microbiol.* 147: 149–154.

Pyörälä S. and S. Taponen. 2009. Coagulase-Negative Staphylococci-Emerging Mastitis Pathogens. *Vet. Microbiol.* 134: 3–8.

Ramisse V., P. Houssu, E. Hernández, F. Denoeud, V. Hilaire, O. Lisanti, F. Ramisse, D. Cavallo and G. Vergnaud. 2004. Variable number of tandem repeats in *Salmonella entérica* subsp. *enterica* for typing purposes. *J. Clin. Microbiol.* 42: 5722–5730.

Ruiz-Romero R., R. Cervantes-Olivares, D. Martínez-Gómez, A. Ducoing-Watty and L. Hernández-Andrade. 2013. Most frequent bacteria isolated from goats' milk in two farms located in Tequisquiapan Querétaro, México. (Principales géneros bacterianos aislados de leche de cabra en dos granjas del municipio de Tequisquiapan, Querétaro, México). *Rev. Mex. Cienc. Pecu.* 4: 93–106.

Sampaio S., S. Simoes, E. Zuniga, P. Melville. A. Bispo, G. Carvalho, F.Gregori and B. Roberti. 2015. Virulence factor genes of *Staphylococcus* spp. isolated from caprine subclinical mastitis. *Microb. Pathog*. 85: 35–39.

Shimizu A., W. Kloos, H. Berkhoff, C. George and D. Ballard. 1997. Pulsed-field electrophoresis of *Staphylococcus hyicus* and *Staphylococcus chromogenes* genomic DNA and its taxonomic, epidemiologic and ecologic applications in veterinary medicine. *J. Vet. Med. Sci.* 59: 443–450.

Schukken Y., R. González., L. Tifosky, H. Schulte, C. Santisteban, F. Welcome. G. Bennet, M. Zurakowski and R. Zadoks. 2009. CNS mastitis: nothing to worry about? *Vet. Microbiol.*134: 9–14.

Stepanovic S., T. Hauschild, L. Dakic, Z. Al-Doori, M. Svabic-Vlahovic, L. Ranin and D. Morrison. 2006. Evaluation of phenotypic and molecular methods for detection of oxacillin resistence in members of the *Staphylococcus sciuri* Group. *J. Clin. Microbiol.* 44: 934–937.

Supré K., F. Haesebrouck, R. Zadoks, M. Vaneechoutte, S. Piepes and S. De Vliegher. 2011.Some Coagulase-Negative *Staphylococcus* Species Affect Udder Health More Than Others. *J. Dairy Sci.* 94: 2329–2340.

Taponen S. and S. Pyörälä. 2009. Coagulase-negative staphylococci as cause of bovine mastitis: not so different from *Staphylococcus aureus*? *Vet. Microbiol.* 34: 29–36.

Taylor J. and F. Breden. 2000. Slipped-strand mispairing and noncontiguous repeats in *Poecilia reticulata* a model for minisatelitte birth. *Genetics* 155: 1313–1320.

Thordberg M., I. Kühn, F. Moller, B. Brandstrom, P. Jonsson and M. Danielson-Tham. 2006. Pheno-and genotyping of *Staphylococcus epidermidis* isolated from bovine milk and human skin. *Vet. Microbiol.* 115: 163–172.

Van Belkum A., S. Scherer, L. Van Alphen and H. Verbrough. 1998. Short-sequence DNA repeats in prokaryotic genomes. *Microbiol. Mol. Biol. Rev.* 62: 275–293.

Vanderhaeghem W., S. Piepers, F. Leroy, E. Van Coillie, F. Haesebrouck and S. De Vliegher. 2015. Identification, typing, ecology and epidemiology of coagulase negative Staphylococci associated with ruminants. *Vet. J.* 203: 44–51.

Vergnaud G. and C. Pourcel. 2009. Multiple locus variable number of tandem repeats analysis. molecular epidemiology of microorganisms. *Methods Mol. Biol.* 551:141–58.

Vilchez G. and G. Alonso. 2009. Alcances y limitaciones de los métodos de epidemiología molecular basados en el análisis de ácidos nucléicos. *Rev. Soc. Ven. Microbiol.* 29: 6–12.

Vogler A., C. Keys, Y. Nemoto, R. Colman, Z. Jay and P. Keim. 2006. Effect of repeats copy number on variable number tandem repeat mutations in *Escherichia coli* O157:H7. *J. Bacteriol.* 188: 4253–4263.

Wang H., B. Diao, Z. Cui, M. Yan and B. Kan. 2016. Genotyping of *Salmonella* Typhi using 8 loci multi locus VNTR analysis. *Gut. Pathog.* 8:14. doi: 10.1186/s13099-016-0094-4.

Zadocks R., and J. L. Watts. 2009. Species Identification of coagulase-negative staphylococci: genotyping is superior to phenotyping. *Vet. Microbiol.* 134: 20–28.

Zaluga J., P. Stragier, J. Van Vaerenberg, M. Maes and P. De Vos. 2013. Multilocus variable number tandem repeats analysis (MLVA) distinguishes a clonal complex of *Clavibacter michiganensis* subsp. *Michiganensis* strains isolated from recent outbreaks of bacterial wilt and canker in Belgium. *BMC Microbiol.* 13:126. doi: 10.1186/1471-2180-13-126.

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ORIGINAL PAPER

Screening and Identification of *Trichoderma* Strains Isolated from Natural Habitats with Potential to Cellulose and Xylan Degrading Enzymes Production

ROMAN MARECIK[1]*, LIDIA BŁASZCZYK[2], RÓŻA BIEGAŃSKA-MARECIK[3], AGNIESZKA PIOTROWSKA-CYPLIK[3]

[1] Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences, Poznań, Poland
[2] Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland
[3] Institute of Food Technology of Plant Origin, Poznań University of Life Sciences, Poznań, Poland

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Abstract

A total of 123 *Trichoderma* strains were isolated from different habitats and tested for their ability to degrade cellulose and xylan by simple plate screening method. Among strains, more than 34 and 45% respectively, exhibited higher cellulolytic and xylanolytic activity, compared to the reference strain *T. reesei* QM 9414. For strains efficiently degrading cellulose, a highest enzyme activity was confirmed using filter paper test, and it resulted in a range from 1.01 to 7.15 FPU/ml. Based on morphological and molecular analysis, the isolates were identified as *Trichoderma*. The most frequently identified strains belonged to *Trichoderma harzianum* species. Among all strains, the most effective in degradation of cellulose and xylose was *T. harzianum* and *T. virens*, especially those isolated from forest wood, forest soil or garden and mushroom compost. The results of this work confirmed that numerous strains from the *Trichoderma* species have high cellulose and xylan degradation potential and could be useful for lignocellulose biomass conversion *e.g.* for biofuel production.

Key words: microorganisms screening, *Trichoderma* species, lignocellulose biomass, cellulolytic activity, xylanolytic activity

Introduction

Lignocellulose is among the most important components of plant biomass. It represents more than half of the globally produced organic matter during photosynthesis. In spite of its high abundance and energetic potential, this resource has not been fully utilized (Piotrowska-Cyplik, Czarnecki, 2003; Sanchez, 2009; Marecik *et al.*, 2012). One of the reasons is a complex structure of plants biomass components, which mainly comprises polymeric compounds, such as cellulose, hemicelluloses, lignin and pectin (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008; Hendriks and Zeeman, 2009; Sarkar *et al.*, 2012). Regrettably, the presence of compounds with such a high degree of polymerization restricts their use as a carbon or energy source for animals and typical fermentation microorganisms. Finding a cheap, and safe for environment method of lignocellulosic biomass degradation would allow increasing feed digestibility and improve effectiveness of livestock production or simple conversion of plant biomass to biofuels (Harris and Ramalingam, 2010; Marecik *et al.*, 2015; Chakdar *et al.*, 2016).

Efficient use of the lignocellulosic resource as a source of renewable energy requires the employment of processes, which lead to the release of monosaccharides. This allows for obtaining substrates, which are easily assimilated by microorganisms and bioconverted to liquid or gaseous fuels, such as ethanol, methanol, hydrogen, methane and others (Saxena *et al.*, 2009). A wide variety of methods can be employed for the degradation of the lignocellulosic complex, including physical, chemical or biochemical treatment. Especially, combined physical and chemical methods allow for rapid and efficient depolymerization of lignocelluloses; however, considerable energy expenditure is required possessing a notable threat to the environment (Kumar *et al.*, 2009; Park and Kim, 2012).

The development of biotechnological hydrolyzation methods for the lignocellulosic complex is considered to be promising. These methods utilize unique properties of microorganisms to degrade different organic and

* Corresponding author: R. Marecik, Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences, Poznań, Poland; **e-mail: roman.marecik@up.poznan.pl**

inorganic or even xenobiotic substances to the simpler or nontoxic ones (Cyplik *et al.*, 2012; Pęziak *et al.*, 2013; Lisiecki *et al.*, 2014). The use of such methods is based on the introduction of specific microorganisms or commercially available enzymatic preparations to the lignocellulosic biomass, what causes release of smaller pentose or hexose components. Enzymatic preparations employed for the decomposition of cellulose or hemicellulose are acquired from the cultivation of selected microbial strains (Aehle, 2007). The complete degradation of cellulose requires cellobiose dehydrogenases (CDHs) enzymes complex containing: endo- and exoglucanases and β-glucosidases. Depending on the producers, CDHs are classified into two classes: class I for CDHs produced by basidiomycetes and class II for CDHs from ascomycetes. Cellobiose dehydrogenases are flavocytochromes and belong to oxidoreductase class of enzymes. The efficient degradation of crystalline cellulose or hemicellulose is strongly related to copper-dependent lytic polysaccharide monooxygenases (LPMOs) (Harreither *et al.*, 2011; Tanx *et al.*, 2015). The preparations used for hemicelluloses hydrolysis are verycomplex, since they usually consists of a mixture of eight enzymes, such as endo-1,4-β-D-xylanase, exo-1,4-β-D-xylanase, α-L-arabinofuranosidase, endo-1,4-β-D-mannase, β-mannosidase, acetyl xylan esterase, α-glucuronidase and α-galactosidase (Clarke, 1997; Jorgensen *et al.*, 2003; Banerjee *et al.*, 2010). However, many different species of microorganisms capable of cellulolytic and hemicellulolytic enzymes synthesis have been discovered, including bacteria and fungi. It is important to note that the efficiency of lignocellulose decomposition is still unsatisfactory (Sun and Cheng, 2002).

Among the microorganisms, which exhibit the ability to produce hemicellulolytic enzymes, the filamentous fungi belonging to the *Trichoderma* genus attract particular attention (Xu *et al.*, 1998). Due to substrate induction, these fungi produce and secrete considerable amounts of enzymes, which belong to cellulases as well as hemicellulases, which is why they are capable of growth under unfavourable environmental conditions (Sandgren *et al.*, 2005). This is a valuable adaptive trait, which allows them to utilize different carbon and energy sources and grow under different temperature regimes, regardless of the presence of light (Polizeli *et al.*, 2005). Due to their various metabolic activity, fungi belonging to the *Trichoderma* genus have found numerous practical applications *e.g.* enzyme producers, used as a biofungicides (Vinale *et al.*, 2006; Wojtkowiak-Gębarowska., 2006; Vinale *et al.*, 2008; Harris and Ramalingam, 2010; Chakdar *et al.*, 2016).

The purpose of this study was to examine the ability of *Trichoderma* fungi isolated from different habitats to production of cellulose and xylan degrading enzyme and determine the activity of those enzymes.

Experimental

Materials and Methods

Fungal collection. The one hundred and twenty-three *Trichoderma* strains, belonging to eleven species or species complex: *Trichoderma atroviride*, *Trichoderma citrinoviride*, *Trichoderma hamatum*, *Trichoderma harzianum, Trichoderma koningii*, *Trichoderma koningiopsis*, *Trichoderma longibrachiatum*, *Trichoderma pseudokoningii*, *Trichoderma viride*, *Trichoderma viridescens* and *Trichoderma virens*, were investigated in this study. The one hundred and seven strains were previously identified by Błaszczyk *et al.* (2011, 2016) and Jeleń *et al.* (2014) and deposited in the collection of the Institute of Plant Genetics, Polish Academy of Science, Poznań, Poland. Ten *Trichoderma* isolates were collected from: wheat kernels (Lublin – AN158 isolate), pieces of decaying wood with white or brown rot (Czerwonak – AN109, AN110 isolates; Golęcin Park, Poznań – AN131 isolate; Strzeszyn Park, Poznań – AN177 isolate; Joniec, Warszawa – AN501 isolate) and mushroom compost used for *Agaricus bisporus* cultivation (Skierniewice – AN186, AN187, AN188 isolates; Poznań – AN204 isolate) in Poland and isolated as described by Błaszczyk *et al.* (2011). Other strains including *T. pseudokoningii* (AN219, ITEM 1416), *T. koningiopsis* (AN222, ITEM2688), *T. harzianum* (AN220, ITEM 1328) and *T. virens* (AN267 – ITEM 1357, AN268 – ITEM 1591, AN269 – ITEM 1594) were kindly supplied by dr. Antonio Logrieco, CNR, ISPA, Bari, Italy. *Trichoderma reesei* QM 9414, sourced from the Czech Collection of Microorganisms (CCM), Brno, Czech Republic was used as the reference strain.

Morphological and molecular analysis. Ten isolates of *Trichoderma* sourced from wheat grains, compost used for mushroom cultivation and pieces of decaying wood collected from the floor of forests and parks in eastern and central Poland were identified morphologically following the procedure described by Gams and Bisset (1998). Colony characteristics were examined from cultures grown on PDA and SNA after 3–7 days at a temperature of 25°C. Microscopic observations were performed from cultures grown on SNA. Molecular species identification was based on the sequencing of two different phylogenetic markers: a fragment of the ITS1-5.8S – ITS2 rRNA region and a fragment of the translation-elongation factor 1-alpha (*tef1*) gene. Mycelium for DNA extraction was obtained as described previously (Błaszczyk *et al.*, 2011). Isolation of total DNA was performed using the CTAB method (Doohan *et al.*, 1998). The ITS1 and ITS2 region of the rDNA gene cluster was amplified using primers ITS4 and ITS5 (White *et al.* 1990). A fragment of the 1.2-kb *tef1* gene was ampli-

fied using primers Ef728M (Carbone and Kohn, 1999) and TEF1LLErev (Jaklitsch *et al.*, 2005). PCR amplification, DNA sequencing and sequence analysis was carried out under the conditions described by Błaszczyk *et al.* (2011). The sequences were identified by BLASTn (http://blast.ncbi.nlm.nih.gov/) as well as TrichOKEY and TrichoBLAST (http://www.isth.info; Druzhinina *et al.*, 2005; Kopchinskiy *et al.*, 2005). The sequences were deposited in the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and listed in Table I.

Cultivation of Trichoderma and induction of enzyme synthesis. For assessing the capability to celululolytic or hemicellululolytic enzyme production, the fungi were cultivated on medium consisting only of carboxymethylcellulose sodium salt (Akzo Nobel Chemicals) or xylan (10 g/l) as a sole source of carbon. Furthermore, the medium contained: $NaNO_3$ – 3 g/l, K_2HPO_4 – 1 g/l, $MgSO_4 \cdot 7H_2O$ – 0.5 g/l, KCl – 0.5 g/l, $FeSO_4 \cdot 7H_2O$ – 0.01 g/l and pH was adjusted to 5.6 ± 0.1. The inducing enzyme synthesis culture was carried out in 300 ml Erlenmeyer flasks, on a rotary shaker (150 rpm) for five days at a temperature of 25 ± 1°C. After the cultivation process the fungal cells were centrifuged (4500 rpm for 10 min) and obtained supernatants containing crude cellulolytic and xylanolytic enzymes were used for determination of the enzymes activity.

Analysis of cellulolytic and xylanolytic activity of Trichoderma fungi – plate method. The analysis of cellulolytic and hemicellululolytic enzymes activity was carried out using the plate screening method described by Hadkin and Anagnostakis (1977). The method is based on the observation of changes (determination of the size of clearance zones), which occur in the solid medium as a result of enzymatic activity. For determination of the cellulolytic activity, the medium including a 1% solution of carboxymethylcellulose sodium salt and 0.1 g/l of chloramphenicol in 2% solution of agar was used. The media were poured into Petri dishes (diameter of 90 mm) and then, after solidification, the central part was removed using a cork borer to create a well. To evaluate the xylanolytic activity the plates were prepared analogously, however a 1% solution of xylan was used instead of carboxymethylcellulose sodium salt.

The cultures of the *Trichoderma* fungi were centrifuged at 4500 rpm for 15 min, and then 200 µl of supernatants were placed in the wells. The plates were incubated at 37°C for 48 h and rinsed with 5 ml of a 1% Lugol's iodine solution. After 15 minutes, the excess of the Lugol's solution was rinsed with 0.1% solution of NaCl. The areas including non-hydrolyzed carboxymethylcellulose sodium salt or xylan were stained with a deep brown colour, whereas the areas in the direct vicinity of the well were characterized by a visible clearance, due to the enzymatic activity. The size of the clearance in each specific sample reflected the activity of cellulolytic or xylanolytic enzymes. The size of the clearance area, which occurred due to the activity of enzymes secreted by a given strain, was compared with the size of the clearance area obtained for the reference strain with known cellulolytic properties – *T. reesei* QM9414 (Sazci *et al.*, 1986).

Analysis of cellulolytic activity of Trichoderma fungi – a blotting filter paper method. The overall cellulolytic activity (FPU) of selected fungal strains was also determined using the method recommended by Ghose (1987). Blotting filter paper stripes (Whatman No. 1) were placed in test tubes and incubated for 60 minutes at 50°C in the presence of 0.1 mol acetate buffer (pH 4.8) and the post-cultivation medium acquired after cultivation of fungi for 5 days. The amount of reducing sugars released into the supernatant was measured by employing the colorimetric method, using 3,4-dinitrosalicylic acid (DNS) (Miller, 1959). The cellulolytic activity of the post-cultivation medium was expressed as FPU (Filter Paper Unit) according to the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987). The amount of the enzyme, which allowed for the release of 1 µmol of glucose during 1 minute, was adapted as one unit of FPU cellulolytic activity.

Statistical analysis of the results. Each experiment of the enzyme activity analysis was carried out in three replicates. The Levene's test (the homogeneity of variance test) and Turkey's test were carried out in order to conduct a statistical verification of the obtained results. The calculations were carried out using Statistica 6.0 software.

Results and Discussion

Trichoderma species identification. Ten isolates of *Trichoderma* from samples of wheat grains, compost used for mushroom cultivation and decaying wood in Poland were identified at the species level based on morphological as well as ITS1, ITS2 and *tef1* sequencing data. Finally, five species or species complex: were found to be: *T. harzianum* species complexes – 3 strains, *T. virens* – 4 strains, *T. viride* – 1 strain, *T. viridescens* – 1 strain and *T. hamatum* – 1 strain. The identification, origin and NCBI GenBank accession numbers of all *Trichoderma* isolates (both of ten isolates identified in this study and isolates previously recognized by Błaszczyk *et al.* (2011, 2016) and Jeleń *et al.* (2014) originating from the different habitats in Poland are given in Table I.

Cellulolytic activity of the studied fungal strains. The studies regarding the cellulolytic activity based on the plate method described by Hadkin and Anagnostakis (1977) revealed that among the 123 strains belonging to the *Trichoderma* genus more than 34% exhibited higher cellulolytic activity compared to the reference

Table I
List of isolates originating from the different habitats identified as the *Trichoderma* species and analyzed for their cellulolytic and xylanolytic activity.

Culture code	Origin		NCBI GenBank Assession No.		Cellulolytic activity (the plate method)	Xylanolytic activity (the plate method)
	Localization	Source	ITS	*tef1*		
T. reesei						
QM9414					1.00	1.00
***T. viride* (A)**						
AN109*, AN176[1]	Central Poland	Forest wood[2]	HQ292923	HQ293010	0.45/0.50	0.70/0.35
AN141			HQ292922	HQ293008	0.75 ± 0.09	1.75 ± 0.27[a]
AN142			HQ292920	HQ293009	2.50 ± 0.31[a]	0.67 ± 0.15
AN179			HQ292924	HQ293011	0.56 ± 0.15	0.55 ± 0.18
AN 235		Forest soil	HQ292921	HQ293013	0.45 ± 0.15	0.75 ± 0.12
AN242	Southern Poland	Forest wood	JX184121	JX184098	0.55 ± 0.18	0.58 ± 0.15
AN244, AN249[1]			JX184122	JX184099	0.46/1.40	0.75/1.42
AN250, AN255[1]			JX184121	JX184098	1.03/0.78	0.77/0.81
AN371			JX184124	JX184100	0.91 ± 0.17	0.92 ± 0.09
AN401			JX184121	JX184099	0.75 ± 0.14	0.47 ± 0.21
***T. iridescent* complex (B)**						
AN93	Central Poland	Forest wood	HQ292927	HQ292995	0.85 ± 0.21	0.73 ± 0.18
AN122			HQ292928	HQ292994	0.79 ± 0.12	0.57 ± 0.19
AN145			HQ292930	HQ292996	0.85 ± 0.17	0.3 ± 0.33
AN148			HQ292933	HQ292999	0.79 ± 0.23	0.44 ± 0.11
AN149			HQ292934	HQ293000	0.67 ± 0.20	0.48 ± 0.14
AN158*	Eastern Poland	Wheat kernels	JX184127	JX184103	0.55 ± 0.12	0.76 ± 0.19
AN227	Central Poland	Forest wood	HQ292936	HQ293001	0.67 ± 0.21	0.57 ± 0.13
AN229			HQ292937	HQ293002	0.83 ± 0.16	0.19 ± 0.08
AN231			HQ292938	HQ293003	0.50 ± 0.23	0.58 ± 0.13
AN245	Southern Poland	Forest wood	JX184127	JX184103	0.50 ± 0.10	0.56 ± 0.16
AN248			JX184128	JX184104	0.63 ± 0.15	0.75 ± 0.23
AN323, AN334, AN405[1]			JX184127	JX184103	0.78/0.85/0.10	0.58/0.58/0.55
***T. vixens* (C)**						
AN68	Eastern Poland	Garden compost	HQ292943	–	2.09 ± 0.27[a]	1.10 ± 0.11
AN69			HQ292944	–	1.60 ± 0.19	3.00 ± 0.27[a]
AN70			HQ292947	–	2.10 ± 0.22[a]	1.10 ± 0.15
AN73			HQ292945	–	2.75 ± 0.31[a]	1.10 ± 0.18
AN74			HQ292946	–	1.25 ± 0.12	1.40 ± 0.17
AN75			HQ292948	–	1.53 ± 0.15	1.10 ± 0.13
AN160		Grass root	HQ292945	–	0.83 ± 0.18	0.55 ± 0.18
AN185	Central Poland	Mushroom compost[3]	HQ292947	–	0.95 ± 0.12	0.55 ± 0.12
AN186*, AN187*,	Eastern Poland		HQ292946	–	0.8/2.6/2.0	1.00/2.25[a]/1.15
AN188* AN204*[1]			HQ292948	–	1.35 ± 0.21	1.45 ± 0.17
AN267 – ITEM 1357	Bari. Italy		–	–	1.25 ± 0.17	0.55 ± 0.20
AN268 – ITEM 1591			–	–	2.50 ± 0.23[a]	2.70 ± 0.32[a]
AN269 – ITEM 1594			–	–	1.6 ± 0.19	1.10 ± 0.12
***T. harzianum* complex (D)**						
AN91	Central Poland	graden Kompost	HQ292860	–	0.75 ± 0.15	1.15 ± 0.11
AN94		forest soil	HQ292873	–	0.80 ± 0.12	3.55 ± 0.27[a]
AN101		forest wood[2]	HQ292868	–	1.60 ± 0.21	2.58 ± 0.23[a]
AN108/AN110*			HQ292869	–	2.80[a]/1.10	1.80[a]/1.40

Table I. Continued

Culture code	Origin		NCBI GenBank Assession No.		Cellulolytic activity (the plate method)	Xylanolytic activity (the plate method)
	Localization	Source	ITS	*tef1*		
AN131*	Central Poland	forest wood[2]	HQ292870	–	1.10 ± 0.16	0.60 ± 0.09
AN132			HQ292867	–	0.78 ± 0.12	0.60 ± 0.07
AN133			HQ292874	–	2.05 ± 0.26[a]	1.70 ± 0.21[a]
AN134			HQ292875	–	1.01 ± 0.11	1.00 ± 0.17
AN135			HQ292876	–	0.81 ± 0.13	1.65 ± 0.14
AN136			HQ292901	–	2.40 ± 0.22[a]	2.04 ± 0.18[a]
AN137			HQ292877	–	1.60 ± 0.13	1.03 ± 0.12
AN138			HQ292861	–	1.00 ± 0.10	1.41 ± 0.16
AN150			HQ292878	–	0.87 ± 0.08	1.67 ± 0.16[a]
AN177*			HQ292883	–	1.25 ± 0.15	1.40 ± 0.11
AN181			HQ292875	–	1.66 ± 0.12[a]	2.25 ± 0.22[a]
AN203		mushroom compost[3]	HQ292879	–	1.66 ± 0.13[a]	1.00 ± 0.18
AN205			HQ292880	–	1.36 ± 0.17	2.45 ± 0.25[a]
AN207			HQ292881	–	1.50 ± 0.13	1.00 ± 0.17
AN211			HQ292882	–	1.10 ± 0.11	0.20 ± 0.11
AN223		Forest soil	HQ292902	–	1.25 ± 0.10	1.05 ± 0.20
AN258		Forest wood	HQ292271	–	0.80 ± 0.08	1.41 ± 0.26
AN260			HQ292884		0.89 ± 0.11	1.20 ± 0.19
AN273			HQ292886	–	1.37 ± 0.19	1.45 ± 0.15
AN275			HQ292888	–	1.20 ± 0.12	1.40 ± 0.11
AN276			HQ292889	–	0.80 ± 0.09	1.05 ± 0.07
AN278			HQ292890	–	0.75 ± 0.10	1.25 ± 0.19
AN282			HQ292891	–	0.30 ± 0.07	1.10 ± 0.12
AN283			HQ292892	–	0.85 ± 0.15	1.27 ± 0.27
AN284			HQ292893	–	1.15 ± 0.20	1.27 ± 0.22
AN285			HQ292894		1.10 ± 0.18	1.28 ± 0.11
AN286			HQ292895	–	1.35 ± 0.29	1.30 ± 0.13
AN349	Southern Poland	Forest wood	JX184111	JX184089	1.05 ± 0.09	1.38 ± 0.24
AN360, AN367, AN373, AN3811			JX184113	JX184090	2.20[a]/1.70[a]/0.70 1.46	1.27/1.40/1.75[a] 1.52
AN220 – ITEM 1328	Bari, Italy		–	–	0.25 ± 0.16	1.60 ± 0.13
***T. hamatum* (E)**						
AN118	Central Poland	Forest wood[2]	HQ292854	–	0.66 ± 0.21	0.61 ± 0.19
AN155	Eastern Poland	Ryder Rhizosphäre	HQ292851	–	0.82 ± 0.18	0.51 ± 0.11
AN175	Central Poland	Forest wood	HQ292854	–	0.53 ± 0.12	0.28 ± 0.10
AN225, AN238		Forest soil	HQ292856	–	0.95 ± 0.08	0.75 ± 0.11
			HQ292853		0.41 ± 0.21	0.84 ± 0.29
AN277, AN279, AN501*[1]		Forest wood	HQ292853	–	0.50/0.18/0.41	2.3[a]/0.72/0.61
AN521	Northern Poland		HQ292856	–	1.05 ± 0.24	0.75 ± 0.15
***T. atroviride* (F)**						
AN19	Central Poland	Forest soil	HQ292786	HQ292963	0.67 ± 0.13	1.04 ± 0.16
AN35		Maize kernels	HQ292787	HQ292953	0.35 ± 0.09	0.80 ± 0.19
AN90		Garden soil	HQ292788	HQ292954	0.49 ± 0.12	0.35 ± 0.12
AN95		Garden compost	HQ292789	HQ292955	0.90 ± 0.17	1.30 ± 0.18
AN96			HQ292790	HQ292956	0.95 ± 0.15	0.70 ± 0.14
AN111		Forest wood	HQ292791	HQ292964	0.37 ± 0.13	0.75 ± 0.21

Table I. Continued

Culture code	Origin		NCBI GenBank Assession No.		Cellulolytic activity (the plate method)	Xylanolytic activity (the plate method)
	Localization	Source	ITS	*tef1*		
AN152	Central Poland	Triticale kernel	HQ292792	HQ292957	0.59 ± 0.19	1.85 ± 0.30[a]
AN153			HQ292793	HQ292958	0.58 ± 0.10	0.57 ± 0.12
AN182		Forest wood	HQ292794	HQ292965	0.57 ± 0.12	0.45 ± 0.23
AN206		Mushroom compost	HQ292804	HQ292960	0.40 ± 0.19	1.02 ± 0.11
AN212			HQ292795	HQ292966	0.68 ± 0.21	1.27 ± 0.21
AN215			HQ292796	HQ292967	1.10 ± 0.16	1.19 ± 0.14
AN224	Southern Poland	Forest wood	HQ292799	HQ292970	0.36 ± 0.09	0.58 ± 0.13
AN240			JX184119	JX184096	0.23 ± 0.19	0.57 ± 0.17
AN287	Central Poland		HQ292798	HQ292969	0.41 ± 0.11	0.58 ± 0.12
***T. longibrachiatum* (G)**						
AN197	Eastern Poland	Mushroom factory	HQ292780	–	0.77 ± 0.19	1.40 ± 022
AN213	Central Poland	Mushroom compost[3]	HQ292781	–	0.50 ± 0.16	2.70 ± 0.31[a]
***T. citrinoviride* (H)**						
AN89	Central Poland	Garden soil	HQ292841	–	0.50 ± 0.14	1.25 ± 0.27
AN98		Forest wood	HQ292843	–	1.55 ± 0.13	1.07 ± 0.19
AN198		Mushroom factory	HQ292845		0.57 ± 0.17	0.47 ± 0.13
AN199			HQ292846	–	0.95 ± 0.12	2.07 ± 0.26[a]
AN201			HQ292849	–	1.10 ± 0.21	2.08 ± 0.29[a]
AN262, AN303, AN393, AN500[1]	Southern Poland	Forest wood	JX184109	–	2.26[a]/0.85/1.75[a] 0.90	2.88[a]/0.61/0.63 0.80
***T. pseudokoningii* (I)**						
AN219 – ITEM1416	Bari. Italy		–	–	1.42 ± 0.19	2.10 ± 0.22[a]
***T. koningii* (J)**						
AN100	Central Poland	Forest wood	HQ292903	HQ292975	0.35 ± 0.17	0.40 ± 0.17
AN105			HQ292905	HQ292977	0.17 ± 0.08	0.58 ± 0.14
AN106			HQ292906	HQ292978	1.16 ± 0.19	0.40 ± 0.16
AN121			HQ292913	HQ292985	0.58 ± 0.11	1.41 ± 0.27
AN128			HQ292918	HQ292989	0.31 ± 0.09	0.48 ± 0.12
AN151			HQ292919	HQ292990	0.47 ± 0.11	0.55 ± 0.12
***T. koningiopsis* (K)**						
AN222 – ITEM 2688	Bari, Italy		–	–	0.20 ± 0.10	0.30 ± 0.12

* The isolates identified in this study by a combination of morphological and molecular analyses
a – Indicate the statistically significant higher values in comparison to the reference strain *T. reesei* (QM 9414) at $P \leq 0.05$
1 – The identical accession numbers refer to identical sequences
2 – The pieces of decaying wood collected from the floor of forests/parks
3 – The compost used for *Agaricus bisporus* cultivation
A. B. C. … – corresponds to particular species presented in fig. 1.

strain *T. reesei* and that these differences were statistically significant ($p \leq 0.05$) (Fig. 1A). *T. harzianum* can be included as a species with high cellulolytic activity. Among the representatives of this species up to 21 out of 39 strains displayed a higher activity compared to the reference *T. reesei* QM9414 strain. The highest activity was observed for strains AN108, AN133, AN136, and AN360. The activity of these strains exceeded the activity of the reference strain by 2.4 times on the average. An activity exceeding 50% was noted for strains AN101, AN137, and AN367. All of these efficient *T. harzianum* strains were isolated from different locations of forest wood. Another species, which included very active strains with regard to degradation of cellulose, was *T. virens,* especially isolated from garden or mushroom compost. Among these species, 12 out of 15 strains were more active compared to the reference strain. The activity exceeding that of the reference strain by 2.6 times was observed for strains AN73, AN187, and AN268. Additionally, the degradation of cellulose

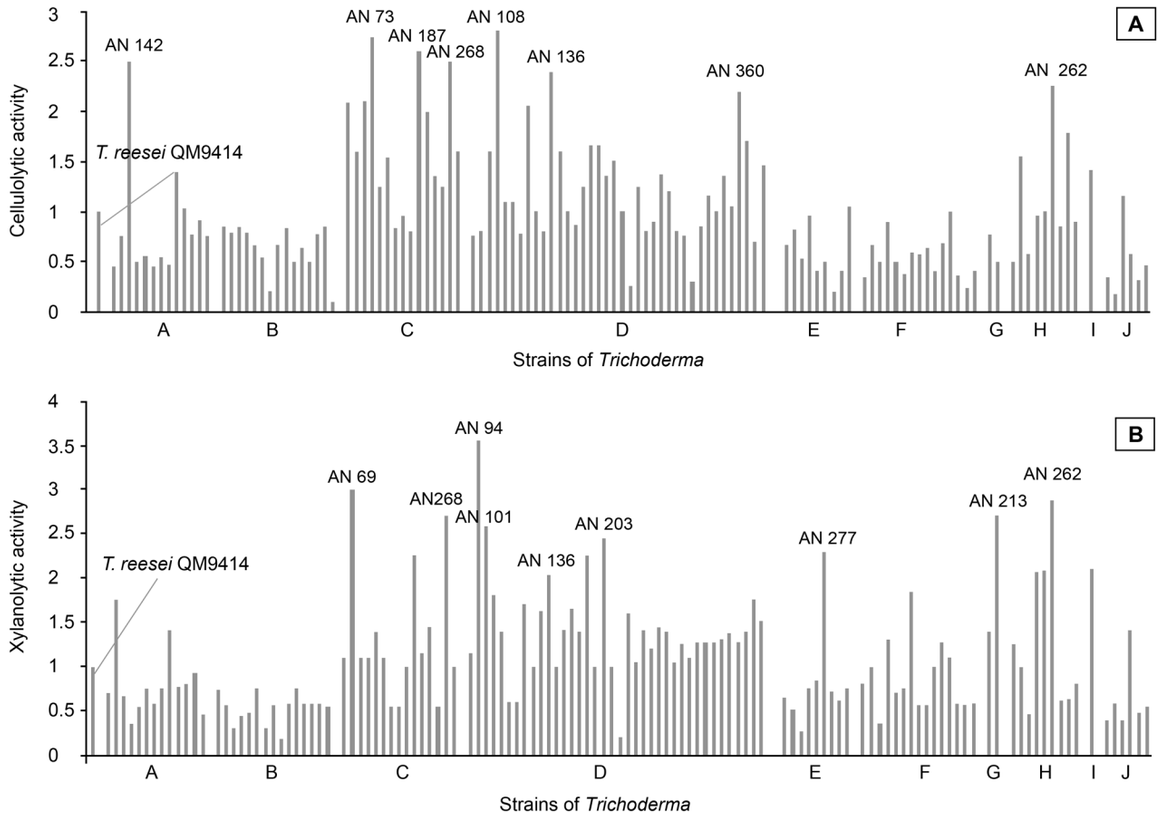

Fig. 1. Cellulolytic (part A) and xylanolytic activity (part B) of the studied fungal strains relative to the reference strain *T. reesei* QM9414 – the plate method analysis. The studied fungal strains belonged to the following species: A – *T. viride*, B – *T. viridescens*, C – *T. virens*, D – *T. harzianum*, E – *T. hamatum*, F – *T. atroviride*, G – *T. longibrachiatum*, H – *T. citrinoviride*, I – *T. pseudokoningii*, J –*T. koningii*.

* The value corresponding to difference in clearing zone diameter between analyzed strains

was approximately twice as efficient for strains AN68, AN70, and AN188. Higher cellulolytic activity compared to the reference *T. reesei* strain was also observed in the case of three strains belonging to the *T. viride* and *T. citrinoviride* species as well as strain from the *T. pseudokoningii* (AN219). Among these species, a particularly high activity was exhibited by AN262 belonging to *T. citrinoviride* species and AN142 belonging to the *T. viride* species, both collected from forest wood. The cellulolytic activity of strains belonging to the remaining species, identified as *T. viridescens*, *T. hamatum*, *T. koningii*, *T. koningopsis* and *T. atroviride* were usually at a much lower level compared to the reference strain. High cellulolytic activity of the selected fungal strains belonging to the *Trichoderma* genus was also confirmed using the blotting filter paper method described by Ghose (1987). The selected strains characterized by the highest cellulolytic activity were presented in Table II.

Table II
Total cellulase activities of selected *Trichoderma* strains measured by the filter paper assay method (FPA).

Cellulolytic activity (FPU/ml)	Strains of *Trichoderma*								
	T. reesei	*T. virens*						*T. harzianum*	
	QM 9414	AN 68	AN 69	AN 73	AN 187	AN 188	AN 268	AN 94	AN 108
	2.11 ± 0.25	4.41 ± 0.31	3.21 ± 0.15	6.05 ± 0.46	5.62 ± 0.41	4.42 ± 0.39	6.36 ± 0.48	1.69 ± 0.09	7.15 ± 0.30

Cellulolytic activity (FPU/ml)	Strains of *Trichoderma*							
	T. harzianum						*T. longibrachiatum*	*T. citrinoviride*
	AN 133	AN 136	AN 181	AN 203	AN 360	AN 367	AN 213	AN 262
	5.31 ± 0.28	6.13 ± 0.55	4.11 ± 0.16	3.33 ± 0.22	5.60 ± 0.25	3.70 ± 0.23	1.01 ± 0.21	4.70 ± 0.34

Xylanolytic activity of the studied fungal strains. The studies regarding the xylanolytic activity of the selected fungal strains belonging to the *Trichoderma* genus revealed that 56 out of 123 studied isolates were characterized by higher activity compared to the reference *T. reesei* strain (Fig. 1B). *T. harzianum* exhibited the highest activity. Up to 31 strains of these species displayed higher activity compared to the reference strain. Among these strains the highest activity was observed for strain AN94 obtained from forest soil, which was capable of degrading xylan over 3.5 times more efficiently compared to the reference strain. A notable xylanolytic activity was also observed in the case of strains AN101 and AN205. These strains exhibited activity, which was over 2.5 times higher compared to the reference strain. *T. citrinoviride* was another species, which included strains with high xylanolytic activity. The strain AN262 that belonged to this species, was capable of degrading xylan over 2 times more efficiently compared to the reference strain. High xylanolytic activity was also noted for AN213, belonging to *T. longibrachiatum* species, AN69 of *T. virens* species and AN277 of *T. hamatum* species.

For both activities analysed, no direct dependence between particular source of fungi strains and their degradative potential was observed; however, the strains isolated from forest wood, forest soil and compost were the most effective.

Filamentous fungi exhibit a broad spectrum of secondary metabolic activity representing important for the people – enzymes or antibiotics production, but also secretion of some dangerous, toxic or cancerogenic substances like mycotoxins (Jae-Hyuk and Keller, 2005; Błaszczyk *et al.*, 2013; Błaszczyk *et al.*, 2016). This is the effect of excellent adaptation ability to different extreme environment condition and the reason why these fungi are very interesting as a source of novel bioactive substances (Altinok, 2009; Chavez, 2015). The strains of *Trichoderma* used in this study were isolated from different habitats: decaying wood, forest soil, garden and mushroom compost, wheat and maize kernels. These habitats are a reach in carbon source but available only for microorganisms able to degrade lignocellulose compounds. This feature is widespread among the different fungi, including *Trichoderma* species (Druzhinina *et al.*, 2010; Amore *et al.*, 2013; Kubicek, 2013). *Trichoderma* genus is very common, diverse and occurs in a wide geographic distribution likewise north regions of Europe; however, the most of *T. harzianum* species identified in this study are uncommon, known mainly from Europe and North America (Jaklitsch *et al.*, 2011; Chavez, 2015; Qin and Zhuang, 2016). The differences in the *Trichoderma* occurrence, related to habitats and geographic regions in Poland, were described also in previous studies (Błaszczyk *et al.*, 2011, 2013, 2016)

Participation of cellulases and hemicellulases in global enzymes market has been increasing year to year. It is the effect of the expanding possibilities of their application in industrial practice (Beg *et al.*, 2000). They may be used as a supplement in animal feeding as well as food or wood industry (Harris and Ramalingam, 2010). Developing biofuel industry (biogas, bioethanol) is also the area of cellulose and hemicellulose enzymes application to increase of the fermentation efficiency (Taherzadeh and Karimi, 2008; Ziemniński *et al.*, 2012; Chakdar *et al.*, 2016). These are the reasons that new and more effective sources of these enzymes are still studied. Many of the microorganisms are saprotrophs and contribute to the decay of organic matter exhibiting the possibility to cellulose and hemicellulose enzymes production (Crowther *et al.*, 2012). However, despite that different microorganisms like bacteria, actinomycetes, yeast or even algae or insects are able to secrete these enzymes, filamentous fungi are especially worth of attention (Polizeli *et al.*, 2005). The genus *Aspergillus* and *Trichoderma* secrete these enzymes directly into the environment at the remarkably higher than other microorganisms efficiency. The ability of different fungi strains belonging to the *Trichoderma* to produce cellulolytic and hemicellulolytic enzymes was extensively studied (Clarke, 1997; Xu *et al.*, 1998; Sandgren *et al.*, 2005; Banerjee *et al.*, 2010). Such enzymes are obtained on industrial scale by aerobic cultivation of fungi, such as *T. reesei* and *Humicola insolens* or from recombinant strains (Liming and Xueliang, 2004; Wilson, 2009). The strains of filamentous fungi isolated from soil, decaying wood and sawdust were analyzed by Inuwa Ja'afaru (2013). Up to 42.6% of the 110 identified isolates belonged to the *Trichoderma* genus. The highest xylanolytic activity was exhibited by *T. viride* Fd18 strain, whereas the highest cellylolytic activity was observed for *Trichoderma* sp. F4 strain. The high potential of fungi belonging to the *Trichoderma* to produce cellulolytic and hemicellulolytic enzymes was confirmed in further studies (Wen *et al.*, 2005; Chandel *et al.*, 2013). Additionally, 23 out of 36 fungal isolates originating from compost also displayed cellulolytic activity. The isolates were identified as *Trichoderma*, *Aspergillus*, *Rhizopus* and *Penicillium* species (Chandel *et al.*, 2013). The ability to synthesize cellulolytic enzymes by the modified *T. reesei* RUT-C30 strain QM 9414 with the use of cow manure as a substrate was confirmed by Wen *et al.* (2005). This strain was characterized by a higher production of cellulose compared to the reference *T. reesei* QM 9414 strain.

In summary, the results obtained in our study confirmed that numerous strains from the *Trichoderma* species are characterized by high lignocellulose degradation potential. The studies performed on forest soil, decaying wood or different kind of compost indicate

a source of effective degraders of cellulose and hemicellulose. Due to potentially benefits related to the production of cellulolytic and hemicellulolytic enzymes and a relatively good growth rate, which is a characteristic trait of such microorganisms; these fungi may be helpful in the industrial practice. For this reason the screening of new producers and study of molecular mechanisms of metabolite secretion regulation should be continued.

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Literature

Aehle W. 2007. Enzymes in industry. Third edition, Wiley-VCH Verlag GmbH & Co. KGaA.

Altinok H.H. 2009. In vitro production of fumonisin B1 and B2 by *Fusarium moniliforme* and the biocontrol activity of *Trichoderma harzianum*. *Ann. Microbiol.* 59(3): 509–516.

Amore A., S. Giacobbe and V. Faraco. 2013. Regulation of cellulase and hemicellulase gene expression in fungi. *Curr. Genomics.* 14: 230–249.

Banerjee S., S. Mudliar, R. Sen, B. Giri, D. Satpute, T. Chakrabarti and R.A. Pandey. 2010. Commercializing lignocellulosic bioethanol: technology bottlenecks and possible remedies. *Biofuels, Bioprod. Bioref.* 4: 77–93.

Beg Q., B. Bhushan, M. Kapoor and G.S. Hoondal. 2000. Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3. *J. Ind. Microbiol. Biot.* 24: 396–402.

Błaszczyk L., D. Popiel, J. Chełkowski, G. Koczyk, G.J. Samuels, K. Sobieralski and M. Siwulski. 2011. Species diversity of *Trichoderma* in Poland. *J. Appl. Genet.* 52: 233–243.

Błaszczyk L., M. Siwulski, K. Sobieralski and D. Frużyńska-Jóźwiak. 2013. Diversity of *Trichoderma* spp. causing *Pleurotus* green mould diseases in Central Europe. *Folia Microbiol.* 58: 325–333.

Błaszczyk L., J. Strakowska, J. Chełkowski, A. Gąbka-Buszek and J. Kaczmarek. 2016. *Trichoderma* species occurring on wood with decay symptoms in mountain forests in Central Europe: genetic and enzymatic characterization. *J. Appl. Genet.* 57: 397–407.

Carbone I. and Kohn L.M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia.* 91: 553–556.

Chakdar H., M. Kumar, K. Pandiyan, A. Singh, K. Nanjappan, P.L. Kashyap and A.K. Srivastava. 2016. Bacterial xylanases: biology to biotechnology. *3 Biotech.* 6(2): 150.

Chandel K., S. Jandaik, V. Kumari, S. Sarswati, A. Sharma, D. Kumar and N. Kumar. 2013. Isolation, purification and screening of cellulolytic fungi from mushroom compost for production of enzyme (cellulase). *Int. J. Curr. Res.* 5(1): 222–229.

Chávez R., F. Fierro, R.O. García-Rico and I. Vaca. 2015. Filamentous fungi from extreme environments as a promising source of novel bioactive secondary metabolites. *Front. Microbiol.* 6: 903.

Clarke A. 1997. Biodegradation of cellulose: enzymology and biotechnology. CRC Press.

Crowther T.W., L. Boddy and T.H. Jones. 2012. Functional and ecological consequences of saprotrophic fungus-grazer interactions. *ISME J.* 6: 1992–2001.

Cyplik P., R. Marecik, A. Piotrowska-Cyplik, A. Olejnik, A. Drozdzyńska and Ł. Chrzanowski. 2012. Biological denitrification of high nitrate processing wastewaters from explosives production plant. *Water Air Soil Poll.* 223(4): 1791–1800.

Doohan F.M., D.W. Parry, P. Jenkinson and P. Nicholson. 1998. The use of species-specific PCR-based assays to analyse *Fusarium* ear blight of wheat. *Plant Pathol.* 47: 197–205.

Druzhinina I.S., A.G. Kopchinskiy, M. Komon, J. Bissett, G. Szakacs and C.P. Kubicek. 2005. An oligonucleotide barcode for species identification in Trichoderma and Hypocrea. *Fungal Genet. Biol.* 42: 813–828.

Druzhinina I.S., M. Komoń-Zelazowska, L. Atanasova, V. Seidl and Ch.P. Kubicek. 2010. Evolution and ecophysiology of the industrial producer *Hypocrea jecorina* (Anamorph *Trichoderma reesei*) and a new sympatric agamospecies related to it. *PLOS ONE.* 5(2): e9191. doi:10.1371/journal.pone.0009191.

Gams W. and J. Bisset. 1998. Morphology and identification of *Trichoderma.* In: Harman G. E. and C. P. Kubicek (eds). *Trichoderma & Gliocladium. Vol. 1.* Taylor and Francis, London.

Ghose T. K. 1987. Measurement of cellulase activities. *Pure & Appl. Chem.* 59: 257–268.

Hadkin L. and S. Anagnostakis. 1977. Solid media containing carboxyl methyl cellulose to detect CM cellulase activity of microorganisms. *J. Gen. Microbiol.* 98: 109–115.

Harreither W., Ch. Sygmund, M. Augustin, M. Narciso, M.L. Rabinovich, L. Gorton, D. Haltrich and R. Ludwig. 2011. Catalytic Properties and Classification of Cellobiose Dehydrogenases from Ascomycetes. *Appl. Environ. Microbiol.* 7(5): 1804–1815.

Harris A.D. and C. Ramalingam. 2010. Xylanases and its application in food industry: a review. *J. Exp. Sciences.* 1(7): 1–11.

Hendriks A. and G. Zeeman. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* 100: 10–18.

Inuwa Ja'afaru M. 2013. Screening of fungi isolated from environmental samples for xylanase and cellulase production. Hindawi Publishing Corporation, *ISRN Microbiol.* Vol. 2013. Article ID 283423.

Jae-Hyuk Y. and N. Keller. 2005. Regulation of secondary metabolism in filamentous fungi. *Annu. Rev. Phytophatol.* 43: 437–458.

Jaklitsch W.M., M. Komon, C.P. Kubicek and I.S. Druzhinina. 2005. *Hypocrea voglmayrii* sp. nov. from the Austrian Alps represents a new phylogenetic clade in Hypocrea/Trichoderma. *Mycologia*. 97: 1365–1378.

Jaklitsch W.M. 2011. European species of Hypocrea part II: species with hyaline ascospores. *Fungal Divers*. 48: 1–250.

Jeleń H., L. Błaszczyk, J. Chełkowski, K. Rogowicz and J. Strakowska. 2014. Formation of 6-n-pentyl-2H-pyran-2-one (6-PAP) and other volatiles by different *Trichoderma* species. *Mycol. Progress.* 3: 589–600.

Jorgensen H., J. Kutter and L. Olsson. 2003. Separation and quantification of cellulases and hemicelulases by capillary electrophoresis. *Anal. Biochem.* 317(1): 85–93.

Kopchinskiy A., M. Komon, C.P. Kubicek and I.S. Druzhinina. 2005. TrichoBLAST: a multilocus database for Trichoderma and Hypocrea identifications. *Mycol. Res.* 109: 657–660.

Kubicek C.P. 2013. Systems biological approaches towards understanding cellulase production by *Trichoderma reesei*. *J. Biotechnol.* 163: 133–142.

Kumar P., D. M. Barrett, M.J. Delwiche and P. Stroeve. 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind. Eng. Chem. Res.* 48 (8): 3713–3729.

Liming X. and S. Xueliang. 2004. High-yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue. *Bioresour. Technol.* 91(3): 259–262.

Lisiecki P., Ł. Chrzanowski, A. Szulc, Ł. Ławniczak, W. Białas, M. Dziadas, M. Owsianiak, J. Staniewski, P. Cyplik, R. Marecik and others. 2014. Biodegradation of diesel/biodiesel blends in saturated sand microcosms. *Fuel.* 116: 321–327.

Marecik R., R. Dembczyński, W. Juzwa, Ł. Chrzanowski and P. Cyplik. 2015. Removal of nitrates from processing wastewater by cryoconcentration combined with biological denitrification. *Desalin. Water Treat.* 54(7): 1903–1911.

Marecik R., J. Wojtera-Kwiczor, Ł. Ławniczak, P. Cyplik, A. Szulc, A. Piotrowska-Cyplik and Ł. Chrzanowski. 2012. Rhamnolipids increase the phytotoxicity of diesel oil towards four common plant species in a terrestrial environment. *Water Air Soil Poll.* 223(7): 4275–4282.

Miller G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426–428.

Park Y.C. and J.S. Kim. 2012. Comparison of various alkaline pretreatment methods of lignocellulosic biomass. *Energy*. 47(1): 31–35.

Pęziak D., A. Piotrowska, R. Marecik, P. Lisiecki, M. Woźniak, A. Szulc, Ł. Ławniczak and Ł. Chrzanowski. 2013. Bioavailability of hydrocarbons to bacterial consortia during Triton X-100 mediated biodegradation in aqueous media. *Acta Biochim. Pol.* 60(4): 789–793.

Piotrowska-Cyplik A. and Z. Czarnecki. 2003. Phytoextraction of heavy metals by hemp during anaerobic sewage sludge management in the non-industrial sites. *Pol. J. Environ. Stud.* 12(6): 779–784.

Polizeli M., A. Rizzatti, R. Monti, H. Terenzi, J.A. Jorge and D. Amorim. 2005. Xylanases from fungi: properties and industrial applications. *Appl. Microbiol. Biotechnol.* 67: 577–591.

Sanchez C. 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnol. Adv.* 27: 185–194.

Sandgren M., J. Ståhlberg and C. Mitchinson. 2005. Structural and biochemical studies of GH family 12 cellulases: improved thermal stability, and ligand complexes, *Prog. Biophys. Mol. Biol.* 89: 246–291.

Sarkar N., S. Kumar, S. Bannerjee and K. Aikat. 2012. Bioethanol production from agricultural wastes: An overview. *Renew. Energ.* 37: 19–27.

Saxena R., D. Adhikari and H. Goyal. 2009. Biomass-based energy fuel through biochemical routes: A review. *Renew. Sust. Energ. Rev.* 13: 168–178.

Sazci A., A. Radford and K. Erenler. 1986. Detection of cellulolytic fungi by using Congo-red as an indicator: a comparative study with the dinitrosalicilic acid reagent method. *J. Appl. Bacteriol.* 61: 559–562.

Sun Y. and J. Cheng. 2002. Hydrolysis of lignocellulosic material for ethanol production: a review. *Bioresour. Technol.* 96: 673–686.

Taherzadeh M. and K. Karimi. 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. *Int. J. Mol. Sci.* 9: 1621–1651.

Than T.-C., D. Kracher, R. Gandini, Ch. Sygmund, R. Kittl, D. Haltrich, B. M. Hällberg, R. Ludwig and Ch. Divne. 2015. Structural basis for cellobiose dehydrogenase action during oxidative cellulose degradation. *Nat. Commun.* 6: 7542. doi: 10.1038/ncomms8542.

Qin W.T. and W.Y. Zhuang. 2016. Seven wood-inhabiting new species of the genus *Trichoderma* (Fungi, *Ascomycota*) in Viride clade. *Sci. Rep.* 6, 27074. doi: 10.1038/srep27074.

Vinale F., R. Marra, F. Scala, E. Ghisalberti, M. Lorito and K. Sivasithamparam. 2006. Major secondary metabolites produced by two commercial *Trichoderma* strains active against different phytopathogens. *Lett. Appl. Microbiol.* 43: 143–148.

Vinale F., K. Sivasithamparam, E.L. Ghisalberti, R. Marra, S.L. Woo and M. Lorito. 2008. *Trichoderma*- plant-pathogen interactions. *Soil Biol. Biochem.* 40: 1–10.

Wen Z., W. Liao and S. Chen. 2005. Production of cellulase by *Trichoderma reesei* from dairy manure. *Bioresour. Technol.* 96(4): 491–499.

White T.J., T. Bruns, S. Lee and J.W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322. In: Innis M.A., D.H. Gelfand, J.J. Shinsky, T.J. White (eds). *PCR protocols: a guide to methods and applications*. Academic, San Diego.

Wilson D.B. 2009. Cellulases and biofuels. *Curr. Opin. Biotechnol.* 20: 295–299.

Wojtkowiak-Gębarowska E. 2006. Mechanizmy zwalczania fitopatogenów glebowych przez grzyby z rodzaju *Trichodrema*. *Post. Mikrobiol.* 45(4): 261–273.

Xu J., N. Takakuwa, M. Nogawa, H. Okada and Y. Morikawa. 1998. A third xylanase from *Trichoderma reesei* PC-3-7. *Appl. Microbiol. Biotechnol.* 49: 718–724.

Ziemniński K., I. Romanowska and M. Kowalska. 2012. Enzymatic pretreatment of lignocellulosic wastes to improve biogas production. *Waste Manag.* 32: 1131–1137.

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ORIGINAL PAPER

The Heavy-Metal Resistance Determinant of Newly Isolated Bacterium from a Nickel-Contaminated Soil in Southwest Slovakia

MATEJ REMENÁR[1], ANNA KAMLÁROVÁ[1], JANA HARICHOVÁ[1], MARCEL ZÁMOCKÝ[1, 2] and PETER FERIANC[1]*

[1] Laboratory of Phylogenomic Ecology, Institute of Molecular Biology of the Slovak Academy of Sciences, Bratislava, Slovakia
[2] Metalloprotein Research Group, Division of Biochemistry, Department of Chemistry, University of Natural Resources and Applied Life Sciences, Vienna, Austria

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Abstract

A bacterial isolate MR-CH-I2 [KC809939] isolated from soil contaminated mainly by high nickel concentrations in southwest Slovakia was previously found carrying *ncc*A-like heavy-metal resistance determinant, marked as MR-CH-I2-HMR [KF218096]. According to phylogenetic analysis of short (696 bp) 16S rDNA (16S rRNA) sequences this bacterium was tentatively assigned to Uncultured beta proteobacterium clone GC0AA7ZA05PP1 [JQ913301]. *ncc*A-like gene product was on the same base of its partial (581 bp) sequences tentatively assigned to CzcA family heavy metal efflux pump [YP_001899332] from *Ralstonia picketii* 12J with 99% similarity. In this study the bacterium MR-CH-I2 and its heavy-metal resistance determinant were more precisely identified. This bacterial isolate was on the base of phylogenetic analysis of almost the whole (1,500 bp) 16S rDNA (16S rRNA) sequence, MR-CH-I2 [MF102046], and sequence for *gyr*B gene and its product respectively, MR-CH-I2-gyrB [MF134666], assigned to *R. picketii* 12J [CP001068] with 99 and 100% similarities, respectively. In addition, the whole *ncc*A-like heavy-metal resistance gene sequence (3,192 bp), marked as MR-CH-I2-nccA [KR476581], was obtained and on the base of phylogenetic analysis its assignment was confirmed to MULTISPECIES: cation efflux system protein CzcA [WP_004635342] from Burkholderiaceae with 98% similarity. Furthermore, although the bacterium carried one high molecular plasmid of about 50 kb in size, *ncc*A-like gene was not located on this plasmid. Finally, the results from RT-PCR analysis showed that MR-CH-I2-nccA gene was significantly induced only by the addition of nickel.

Key words: 16S rRNA (16S rDNA), DNA gyrase subunit B, heavy-metal resistance determinant, high molecular plasmid, nickel-contaminated soil, RT-qPCR

Introduction

Bacterial communities are known to reflect their microenvironmental conditions by readily responding at extremely fast rates to environmental and pollution changes (Bell *et al.*, 2009; Thiyagarajan *et al.*, 2010; Ławniczak *et al.*, 2016; Sydow *et al.*, 2016). However, human activities have dramatically changed the composition and organisation of soils. Among wastes with increased concentrations of contaminants as a result of different anthropogenic activities heavy metals occur frequently (Liu *et al.*, 2005). Moreover, their contribution in overall contamination of soils in Europe is of about 35%, what reveals a greater fraction in comparison to mineral oils (Panagos *et al.*, 2013; Sydow *et al.*, 2017). Although some of heavy metals are necessary in trace amounts for a variety of metabolic processes in the cell but in high concentrations they react to form toxic compounds that can cause its damage (Nies, 1999). Most common heavy metals that were found at contaminated sites are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni) (Gwrtac, 1997; Wuana and Okieimen, 2011; Tóth *et al.*, 2016). Because of their high degree of toxicity, arsenic, cadmium, chromium, lead, and mercury rank among the priority metals that are of public health significance (Tchounwou *et al.*, 2012). Once in the soil, heavy metals are adsorbed by initial fast reactions (minutes, hours), followed by slow adsorption reactions (days, years) and are, therefore, redistributed into different chemical forms with varying bioavailability, mobility, and toxicity (Shiowatana *et al.*, 2001). Although bacteria have been interacting with heavy metals in their natural environments to various extents since their

* Corresponding author: P. Ferianc, Institute of Molecular Biology of the Slovak Academy of Sciences, Bratislava, Slovakia; **e-mail: peter.ferianc@savba.sk**

early evolutionary history, heavy-metals-contaminated soil for long time caused selective pressure on microorganisms, which are forced to respond to these changes in environment by selection of heavy-metal-resistant bacteria. These bacteria have evolved several mechanisms that regulate metal ion accumulation to avoid heavy metal toxicity for the cell. The best-known mechanisms of heavy-metal resistances include permeability barriers, intra- and extra-cellular sequestration, efflux pumps, enzymatic detoxification, and reduction (Nies, 1999). The most well characterized operons conveying resistance against heavy metals in Gram-negative bacteria are the *czc* (cobalt-zinc-cadmium resistance) and *cnr* (cobalt-nickel resistance) operons from *Cupriavidus metallidurans* CH34 (Mergeay *et al.*, 2003), the *ncc* (nickel-cobalt-cadmium resistance) and *nre* (nickel resistance) systems from *Achromobacter xylosoxidans* 31A (Schmidt and Schlegel, 1994) and *czn* (cadmium-zinc-nickel resistance) operon from Helicobacter pylori (Salvador *et al.*, 2007). In Gram-positive bacteria, the *cad* operon from *Bacillus* and *Staphylococcus* members has been well studied (Silver and Phung, 1996). In both Gram-positive and Gram-negative bacteria the *ars* operons from *Escherichia coli* (Mobley *et al.*, 1983; Saltikov and Olson, 2002) and *Staphylococcus* strains (Ji and Silver, 1992; Rosenstein *et al.*, 1992), and the *mer* systems from *E. coli* (Nascimento and Chartone-Souza, 2003) and *Bacillus* populations (Bogdanova *et al.*, 1998) have been characterized. In addition, the cyanobacterial *smt* locus from *Synechococcus* PCC 7942 also contains a well-characterized heavy metal resistance system (Erbe *et al.*, 1995).

In our previous work, a few of hardly cultivable and previously uncultured bacterial isolates from toxic-metal contaminated soil were cultivated, partly identified and characterised by using a diffusion chamber approach. Obtained results showed that all these isolates were resistant to nickel, cobalt, zinc, copper and cadmium ions and that this resistance in majority of β- or γ-Proteobacteria was mediated via a system of transmembrane metal pumps carried by these bacteria (Remenár *et al.*, 2015). These efflux systems are represented mainly by CBA efflux pumps driven by proteins of the resistance-nodulation-cell division superfamily, P-type ATPases, cation diffusion facilitator and chromate proteins, NreB- and CnrT-like resistance factors. Some of these systems are widespread and serve in the basic defence of the cell against superfluous heavy metals, but some are highly specialized and occur only in a few bacteria (Nies, 2003).

In detail, CBA transporters are three-component protein complexes that span the whole cell wall of Gram-negative bacteria. The most important component of the transporter is an RND protein that is located in the inner membrane. It mediates the active part of the transport process, determines the substrate specificity and is involved in the assembly of the trans-envelope protein complex. The RND protein family was first described as a related group of bacterial transport proteins involved in heavy metal resistance (*C. metallidurans*), nodulation (*Mesorhizobium loti*) and cell division (*E. coli*) (Saier *et al.*, 1994). The RND protein is usually accompanied by the membrane fusion protein (MFP) (Saier *et al.*, 1994) and outer membrane factor (OMF) (Paulsen *et al.*, 1997; Johnson and Church, 1999). OMF and MFP proteins have a rather static function during CBA-mediated trans-envelope efflux. These three proteins form an efflux protein complex that may export its substrate from the cytoplasm, the cytoplasmic membrane or the periplasm across the outer membrane directly to the outside of the cell (Zgurskaya and Nikaido, 1999a; 1999b; 2000a; 2000b). In bacteria and archaea, CBA transporters are involved in transport of heavy metals, hydrophobic compounds, amphiphiles, nodulation factors and proteins (Tseng *et al.*, 1999). In addition, these transport systems could remove cations even before they have the opportunity to enter the cell and could mediate 10 further export of the cation that had been removed from the cytoplasm by other efflux systems (Nies, 2003).

The best-characterized CBA transporter is the CzcCBA complex from *C. metallidurans* CH34. The *czc* determinant encodes resistance to Cd^{2+}, Zn^{2+} and Co^{2+} by metal-dependent efflux driven by the proton motive force (Nies, 1995). Ni^{2+} and Co^{2+} are, in some occasions, exported by the same CBA transporters as Zn^{2+} and Cd^{2+} (for example NccCBA from *A. xylosoxidans* 31A and CzcCBA from *C. metallidurans* CH34) (Schmidt and Schlegel, 1994; Legatzki *et al.*, 2003).

Thus, in our studies, we wanted to accurately identify a newly isolated bacterium tentatively assigned to uncultured betaproteobacteria and its heavy-metal-resistance gene product because we expected that such bacterium isolated from extreme environment could serve as a specific soil bacterial strain carrying heavy-metal-resistance gene product that facilitates the cells to survive in soil contaminated with nickel and also containing other metals, such as cobalt, zinc, iron, copper and cadmium.

Experimental

Materials and Methods

Isolation and cultivation of bacterium. Bacterium MR-CH-I2 was isolated by diffusion chamber approach (Kaeberlein *et al.*, 2002) with some modifications (Remenár *et al.*, 2015) from farmland near the town of Sereď (48°16′59″ N, 17°43′35″ E) in southwest

Slovakia. The sampling site was situated near a dump containing heavy-metal-contaminated waste. Investigated field site contained high concentrations of nickel (2109 mg/kg), slightly above the natural occurrence of cobalt (355 mg/kg) and zinc (177 mg/kg), even too low concentration of iron (35.75 mg/kg) for a normal soil and not a toxic amount of copper (32.2 mg/kg) and cadmium (< 0.25 mg/kg). The content of heavy metals in the soil sample was measured using an atomic absorption spectrometer (PerkinElmer model 403, USA) (Karelová *et al.*, 2011). The site is according to environmental monitoring of Slovakia a part of strongly disturbed environment (Bohuš and Klinda, 2010).

Bacterium MR-CH-I2 was cultivated on LB (Luria-Bertani) agar plates aerobically at 30°C for 24 h and independently growing colonies were used for further analysis.

DNA extraction. Bacterial DNA from bacterial MR-CH-I2 cells was isolated using the DNeasy purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as described in Karelová *et al.* (2011). The resulting high-molecular-weight DNA was stored at –20°C and was used as a template in appropriate PCR experiments.

Detection of 16S rRNA (16S rDNA) and DNA gyrase subunit B (*gyr*B) genes. DNA extracted from bacterium MR-CH-I2 was used in PCR either with universal 16S rRNA gene primers (27F, 1492R) or with universal degenerate *gyr*B primer set (UP-1 and UP-2r) (Table I). Each 20 µl reaction mixture contained 1 µl (10 ng) of the DNA template, 2.0 µl 10× AccuPrime *Pfx* Reaction mix (Invitrogen, USA), 1.25 U AccuPrime *Pfx* DNA polymerase (Invitrogen, USA) and 0.5 µM of each primer. PCRs were performed in a thermal cycler

Table I
Primer sets used in this study.

Probes	Sequence	Description[a]
27F	5' AGAGTTTGATCCTGGCTCAG 3'	16S rDNA universal primers, positions 8–27 and 704–685 and 1512–1492, resp. in the *E. coli* K12 [NC_000913] numbering system; (Lane, 1991)
1492R	5' ACGGCTACCTTGTTACGACTT 3'	
2555nccAF	5' AGCCG (C,G) GA (C,G) AACGG CAAGCG 3'	2536–2555 and 3136–3117 degenerative nccA primers, positions on plazmid p9 in the *Achromobacter xylosoxidans* 31A [L31363] numbering system; (Karelová *et al.*, 2011)
3117nccAR	5' CCGATCACCACCGT (T,C) GC CAG 3'	
nccA9F	5' ACGTATCATTAGTTTCGCCA 3'	1861923–1861904 and 1859057–1859076 *ncc*A primers, positions on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; This work
nccA2875R	5' ATCGGATAAACGACAGCATC 3'	
nccA1244F	5' GCTCTCGAAAGAGGAAGGCA 3'	1862989–1862970 and 1861746–1861765 *ncc*A primers, positions on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; This work
nccA1244R	5' TTCGGTTTCGAGCGGTGAAT 3'	
nccA642F	5' GCTAGTCTTCACGGGCATT 3'	1859211–1859193 and 1858570–1858589 *ncc*A primers, positions on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; This work
nccA642R	5' GCTCTTCGTCATGACACCAC 3'	
nccA923F	5' GGTCGCTTCCATTAACCG 3'	1860996–1860979 and 1860074–1860091 *ncc*A primers, positions on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; This work
nccA923R	5' GATCGGATGCAATCTCCG 3'	
nccA-F	5' GTCGCCTTGTTCATCGG 3'	1860425–1860409 and 1860301–1860319 *ncc*A primers, positions on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; This work
nccA-R	5 GCAAACGTCAATACAACGG 3'	
gdhA-F	5' CGTACTCAATGAACGAAGGC 3'	388722–388741 and 388866–388850 *gdh*A primers, positions on chromosome in the *Ralstonia picketii* 12D [CP001644] numbering system; This work
gdhA-R	5' TCGATGCCGAGATTGCG 3'	
UP1	5' GAAGTCATCATGACCGTTCTG CA(TC)GC(TCAG)GG(TCAG)GG (TCAG)AA(AG)TT(TC)GA 3'	*gyr*B gene primers, positions 91–104 and 495–509 amino acid residues (the numbering corresponds to that of the *E. coli* K12 protein [(GYRB_ECOLI in the SWISS-PROT database)]) (Yamamoto and Harayama, 1995)
UP2r	5' AGCAGGGTACGGATGTGCGAG CC(AG)TC(TCAG)AC(AG)TC(TC AG)GC(AG)TC(TCAG)GTCAT 3'	
UP-1S	5' GAAGTCATCATGACCGTTCT GCA 3'	
UP-2Sr	5' AGCAGGGTACGGATGTGCG AGCC 3'	

[a] Numbers in parenthesis indicate the GenBank accession number.

(LabCycler, Goettingen, Germany) with the following cycling conditions: 2 min of denaturation at 95°C, 35 cycles of 20 s at 95°C, 30 s at 53°C (16S rRNA) or 58°C (*gyr*B), 1 min 40 s (both, 16S rRNA and *gyr*B) at 68°C, and a final cycle of extension at 68°C for 10 min. PCR products were separated by electrophoresis in a 1% (w/v) agarose gel (Merck, Germany) and stained with GelRed Nucleic Acid Gel Stain (Biotium, USA). DNA bands, approximately 1,500 or 1,200 bp in size for the 16S rRNA and *gyr*B genes respectively, were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Detection of complete *ncc*A-like gene and its sequencing strategy. DNA extracted from bacterium MR-CH-I2 was used in PCRs with subsequent primer sets:

i) *ncc*A-like gene fragment amplification with nccA9F and nccA2875R primers located in early and terminal parts of the gene, respectively (Table I);
ii) amplification of beginning of *ncc*A-like gene with nccA1244F and nccA1244R primers located before the gene and in known early part of the gene, respectively (Table I);
iii) amplification of terminal part of *ncc*A-like gene with nccA642F and nccA642R primers located in earlier known terminal part and outside of the gene, respectively (Table I);
iv) amplification of middle part of *ncc*A-like gene with nccA923F and nccA923R primers located in earlier known early and terminal parts of the gene, respectively (Table I).

Each 50 µl reaction mixture contained 1 µl (10 ng) of the DNA template, 5.0 µl 10 × Taq buffer (Qiagen, Hilden, Germany), 2.5 U Taq DNA polymerase (HotStar; Qiagen, Hilden, Germany), 1.5 mM $MgCl_2$, 400 nM of each dNTP and 0.5 µM of each primer. PCRs were performed in a thermal cycler (LabCycler, Goettingen, Germany) with the following cycling conditions: 15 min of denaturation at 95°C, 35 cycles of 1 min at 95°C, 1 min at 55°C (i), 58°C (ii), 57°C (iii) or 56°C (iv), 5 min 40 s (i), 2 min 36 s (ii), 1 min 20 s (iii) or 1 min 50 s (iv) at 72°C, and a final cycle of extension at 72°C for 8 min. PCR products were separated by electrophoresis in a 1% (w/v) agarose gel (Merck, Germany) and stained with GelRed Nucleic Acid Gel Stain (Biotium, USA). DNA bands, approximately 2,866, 1,244, 642 or 923 bp in size for i) – iv) gene fragments respectively, were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Sequencing of 16S rRNA (16S rDNA), *gyr*B and *ncc*A-like amplicons. Subsamples of either purified 16S rRNA (16S rDNA), *gyr*B (*gyr*B was sequenced with UP-1S and UP-2Sr primers (Table I)) or all *ncc*A-like amplicons from isolate were sequenced by GATC Biotech, Constance, Germany.

Bacterial strain and *ncc*A-like gene product identifications and phylogenetic analysis. Bacterial strain identification, identification of *ncc*A-like gene products and phylogenetic analysis were performed as described in Karelová *et al.* (2011) with following modifications: multiple sequence alignments and phylogenetic trees were constructed with the MEGA software (version 6, Tamura *et al.*, 2011). Maximum likelihood method with 100 bootstrap replications was chosen with Tamura-Nei model of substitutions and the resulting tree was presented with the Tree Explorer of the MEGA package.

Plasmid DNA purification and electroporation. Plasmid DNA from bacterial MR-CH-I2 cells was purified using the Wizard minipreparation kit (Promega, USA) according to the manufacturer's instructions. Plasmid transformations were performed by electroporation on equipment ECM 630 (BTX Harvard apparatus, USA) after one impulse effect (2.5 kV, 200 Ω, 25 µF).

Pulsed-field gel electrophoresis (PFGE). PFGE was performed in equipment ROTAPHOR (Biometra, Germany) as described in Nováková *et al.* (2013) with the following modifications: electrophoresis ran at 10°C for 18 h with constant interval of about 2 s, under linear angle from 130° to 110° and linear voltage from 130 V to 90 V.

Bacterial cells preparation for *ncc*A-like gene expression analysis. Bacterial culture MR-CH-I2 was grown aerobically in liquid Luria-Bertani (LB) medium in Erlenmeyer flasks in a rotary shaker (90 rpm) at 30°C. When cultures reached an optical density at 420 nm (OD_{420}) of 0.5, five heavy metals were added to a previously optimized final concentration of 250 µg/ml Ni^{2+}, 100 µg/ml Cd^{2+}, 50 µg/ml Zn^{2+}, 25 µg/ml Co^{2+} and 10 µg/ml Cu^{2+}, respectively (Remenár *et al.*, 2015). Appropriate aliquots of bacterial cultures were withdrawn from culture either before heavy metals addition (control sample) or 0, 2, 4, 6 and 8 h after heavy metal additions.

Total RNA isolation and purification and cDNA preparation. Total RNA from bacterial MR-CH-I2 cells, cultivated in the presence of different heavy metals and without heavy metal additions (control sample), was isolated and purified using the RiboPure Bacteria Kit (Ambion, USA) according to the manufacturer's instructions. cDNA was prepared from isolated and purified RNA using ImProm-II Reverse Transcription System (Promega, USA) according to the manufacturer's instructions.

Real-time PCR. cDNA prepared from RNA of bacterium MR-CH-I2 was used in Real-time PCR either with *ncc*A gene primers (nccA-F, nccA-R) or with housekeeping *gdh*A primer set (gdhA-F and gdhA-R) (Table I) generating approximately 125 bp or 145 bp products in size respectively. Each 20 µl reaction mixture contained 2 ng of the cDNA template, 4.0 µl 5 × HOT FIREPol EvaGreen qPCR SuperMix (Solis Bio-

Dyne, Esthonia) and 0.5 μM of each primer. Reaction was performed in a thermal cycler ABI 7900HT FAST Real-Time PCR System (Life Technologies, USA) with the following cycling conditions: 12 min of denaturation at 95°C, 40 cycles of 15 s at 95°C, 20 s at 55°C and 20 s at 72°C. Results were evaluated using SDS software of the ABI 7900HT device.

Nucleotide sequence accession numbers. The sequences generated in this study have been deposited in the GenBank database under accession number MF102046 for MR-CH-I2 16S rRNA (16S rDNA) gene, MF134666 for MR-CH-I2-gyrB gene and KR476581 for complete MR-CH-I2-nccA gene of MR-CH-I2.

Results and Discussion

Identification of the specific heavy-metal resistance bacterium. To identify unequivocally previously isolated heavy-metal resistant bacterium we performed a phylogenetic analysis of almost the whole (1,500 bp) 16S rDNA (16S rRNA) sequence and sequences for *gyr*B gene and its product, respectively. The results from these analyses showed that the bacterial isolate, marked as MR-CH-I2 [MF102046] and MR-CH-I2-gyrB [MF134666], respectively was assigned to *Ralstonia picketii* 12J chromosome 1 [CP001068] with 99 and 100% similarities, respectively (Figs. 1, 2). This

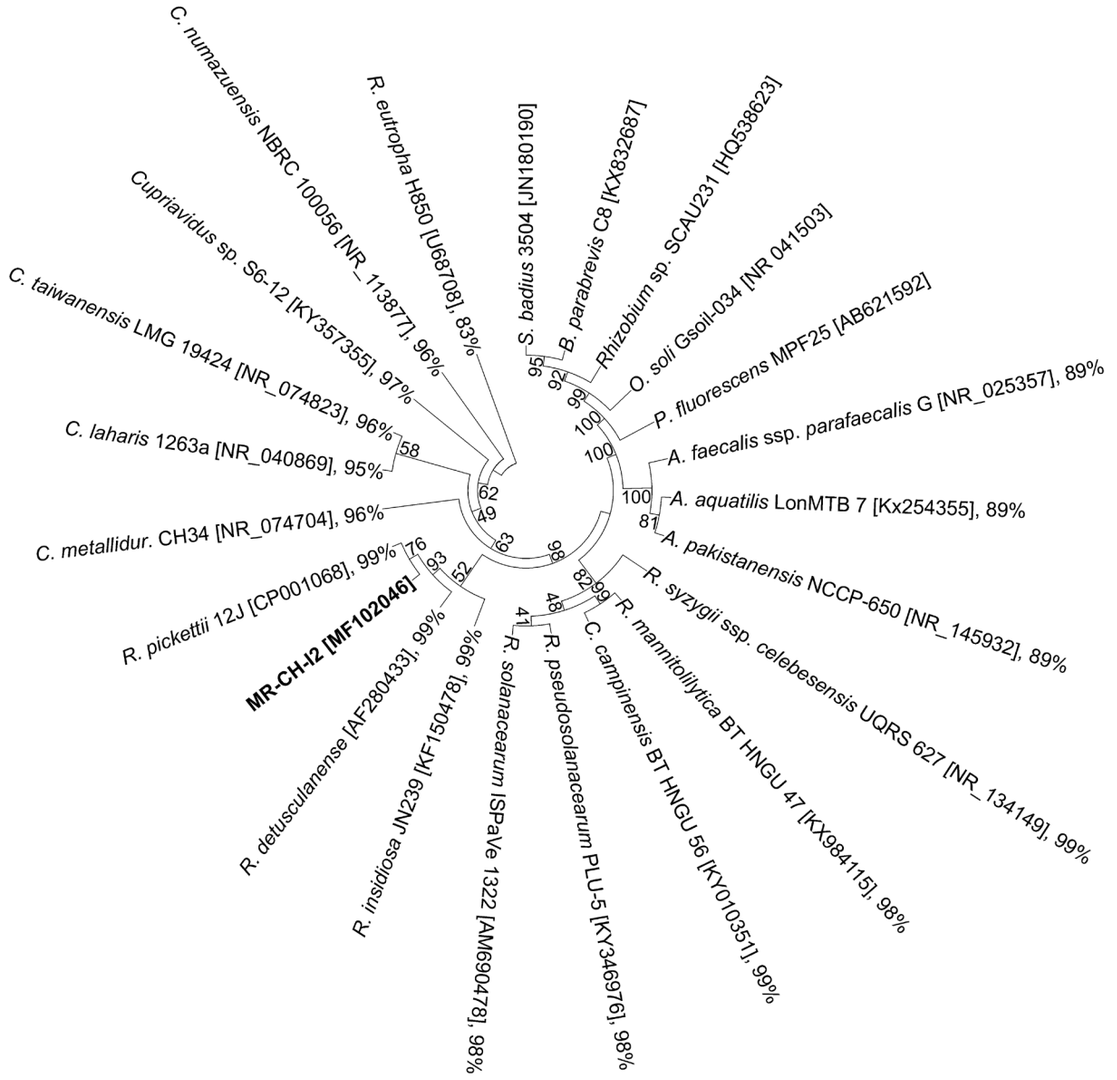

Fig. 1. Unrooted phylogenetic tree obtained by the maximum likelihood method with 100 bootstrap replications showing phylogeny of 16S rRNA (16S rDNA) gene sequences of MR-CH-I2 isolate (in bold) and members of the genera *Ralstonia*, *Cupriavidus* and *Alcaligenes*, respectively. *Rhizobium* sp. SCAU231 [HQ538623], *Pseudomonas fluorescens* strain MPF25 [AB621592], *Streptomyces badius* strain 3504 [JN180190], *Olivibacter soli* strain Gsoil 034 [NR_041503] and *Brevibacillus parabrevis* C8 [KX832687] were used as outgroup. Numbers in square brackets indicate the GenBank accession number and similarity to closest relative is shown after the clone designation. Sequences of about 1 500 bp in length were aligned with ClustalW.

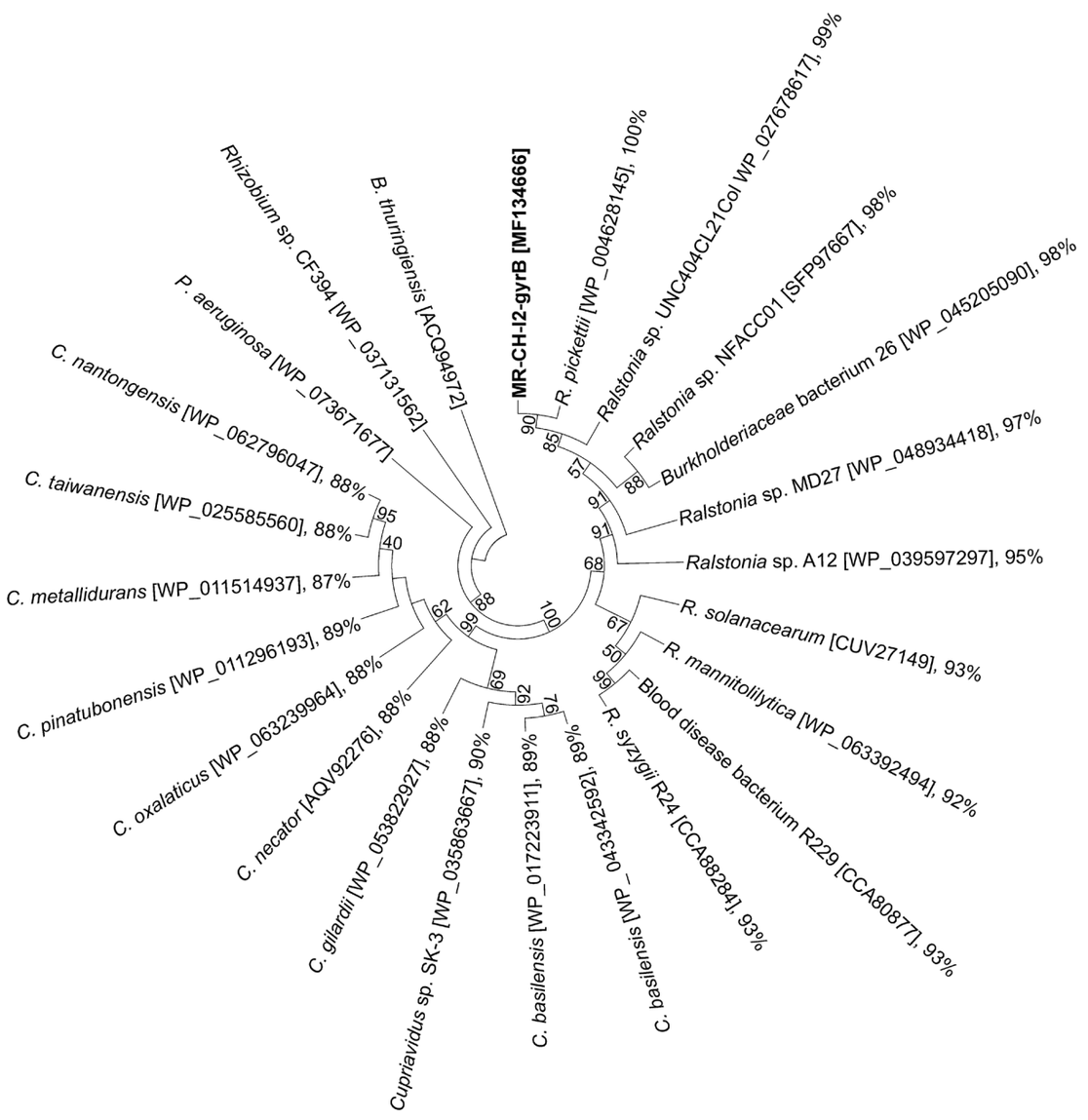

Fig. 2. Unrooted phylogenetic tree obtained by the maximum likelihood method with 100 bootstrap replications showing phylogeny of partial *gyr*B sequences based on 399 presented amino acid sites translated from DNA of MR-CH-I2 isolate (in bold) and members of the genera *Ralstonia*, *Cupriavidus* and family Burkholderiaceae, respectively. *Rhizobium* sp. CF394 [WP_037131562], *Pseudomonas aeruginosa* [WP_073671677] and *Bacillus thuringiensis* [ACQ94972] served as outgroup. Numbers in square brackets indicate the GenBank accession number and similarity to closest relative is shown after the clone designation. Sequences were aligned with ClustalW.

bacterium was firstly found inside of bacterial community isolated by using diffusion chamber methods from strongly disturbed environment mainly by high nickel concentrations in southwest Slovakia, and marked as MR-CH-I2 [KC809939] and tentatively assigned to Uncultured beta proteobacterium clone GC0AA7ZA05PP1 [JQ913301] on a β-Proteobacteria branch of phylogenetic tree (Remenár *et al.*, 2015). Several representatives of this class of Proteobacteria are often highly versatile in their degradation capacities and many of them are found in environmental samples, such as waste water or soil (Stackebrandt *et al.*, 1988). But only one of them, *R. pickettii* is able to survive as an oligotrophic organism in areas with a very low concentrations of nutrients (Adley *et al.*, 2007). It was found in moist environments such as soils, rivers and lakes (Coenye *et al.*, 2003). Several strains have shown an ability to survive in environments highly contaminated with metals such as copper (Cu), nickel (Ni), iron (Fe) and zinc (Zn). The ability to persist in these harsh conditions makes *R. picketti* a unique candidate for bioremediation (Fett *et al.*, 2003). *R. pickettii* can also break down several aromatic hydrocarbons or volatile organic compounds such as cresol, phenol

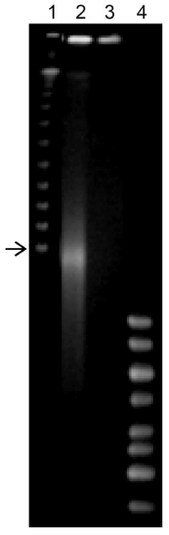

Fig. 3. Pulsed-field gel electrophoresis analysis of high molecular plasmids from bacterium MR-CH-I2.

Legends: Lane 1 = mass standard (Lambda Ladder PFGE Marker); lane 2 = plasmid from isolate MR-CH-I2; lane 3 = control sample (without plasmid); lane 4 = mass standard (1 Kb DNA Ladder). The arrow indicates the band of about 50 kb in size.

and toluene and it is able to exploit this resource by using the hydrocarbons as both a source of carbon and energy (Adley *et al.*, 2007). Thus, the identification of *R. pickettii* among β-Proteobacteria representatives isolated from heavy-metal contaminated soil by using of diffusion chamber is not surprising.

In addition, pulsed-field gel electrophoresis (PFGE) showed that the bacterium MR-CH-I2 carried a unique high molecular weight plasmid of about 50 kb in size (Fig. 3). It is known that there are two separate strains of *R. pickettii*, 12D and 12J which vary significantly in their entire genome size, 3.5 Mb and 3.0 Mb respectively. In spite of the fact that their rRNA sequences are identical, there are significant differences in their genomic structures (http://genome.jgi-psf.org/ralpd/ralpd.home.html). While both, 12D and 12J strains contain two circular chromosomes 3,647,724 bp and 1,323,321 bp in size and 3,942,557 bp and 1,302,228 bp in size respectively, the 12D strain contains three circular plasmids 389,779 bp, 273,136 bp and 51,398 bp in size whereas the 12J strain has only one circular plasmid that is 80,934 bp in size (http://www.expasy.ch/sprot/hamap/RALP1.html; http://expasy.org/sprot/hamap/RALPJ.html). In spite of the fact that there are some discrepancies about the plasmid size between our data and cited one, our bacterium carried only one plasmid, thus this result also endorses the correct assignment of bacterium MR-CH-I2 to *R. pickettii*, strain 12J. In addition, another *R. picketii* strains were found which possesses a unique plasmid of about 50 kb in size as well (http://genome.jgi-psf.org/ralpd/ralpd.home.html).

The correct assigment of *ncc*A-like heavy-metal resistance determinant. Except the presence of one plasmid of a higher molecular weight, MR-CH-I2 has been found to carry *ncc*A-like heavy-metal resistance determinant, firstly marked as MR-CH-I2-HMR [KF218096]. On the base of phylogenetic analysis of its partial (581 bp) sequence it was tentatively assigned to CzcA family heavy metal efflux pump [YP_001899332] from *R. picketii* 12J with 99% similarity (Remenár *et al.*, 2015). According to sequencing strategy of PCR amplicons covering a complete encoding area of *ncc*A-like heavy-metal resistance gene and its neighbouring sequences partly before and partly after of its encoding area (in detail described in "Materials and methods" section) (Fig. 4), the whole *ncc*A-like heavy-metal resistance gene sequence was obtained and marked as MR-CH-I2-nccA [KR476581] of about 3,192 bp in length and of 1,063 amino acids (115,620 Da in molecular weight), respectively. The results from following phylogenetic analysis of complete *ncc*A-like heavy-metal resistance gene have confirmed its assignment to CzcA family heavy metal efflux pump [WP_004635342] from *R. picketii* 12J with 98% similarity (Fig. 5). However, the presence of this heavy-metal resistance gene on the plasmid was not confirmed. WP_004635342 is a new term replacing YP_001899332. Thus previous protein reference sequence YP_001899332 has been replaced by WP_004635342.1. The sequence YP_001899332 for "CzcA family heavy metal efflux pump [*R. picketii* 12J]"

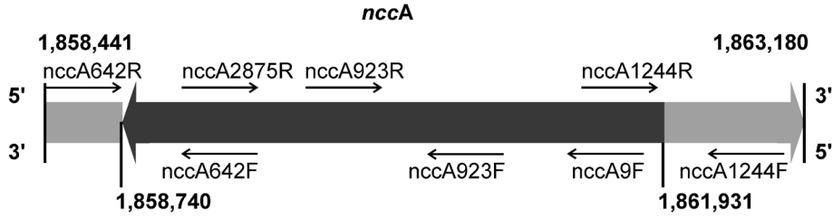

Fig. 4. The whole MR-CH-I2-nccA [KR476581] gene sequencing strategy of MR-CH-I2 isolate (cf. Detection of complete *ncc*A-like gene and its sequencing strategy in section Materials and methods and Table I).

Legends: Numbers in bold indicate positions of the MR-CH-I2-nccA gene (dark-skinned grey arrow) and its neighbourhood areas (light grey arrow) on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; thin arrows indicate positions of appropriate primers on the MR-CH-I2-nccA gene and its neighbourhood areas; nccA1244F and nccA1244R primers were used for sequencing of the beginning of the MR-CH-I2-nccA gene; nccA9F and nccA2875R primers were used for sequencing of the beginning and terminal parts of the middle area of the MR-CH-I2-nccA gene; nccA923F and nccA923R primers were used for sequencing of the middle parts of the middle area of the MR-CH-I2-nccA gene; nccA642F and nccA642R primers were used for sequencing of terminal part of the MR-CH-I2-nccA gene.

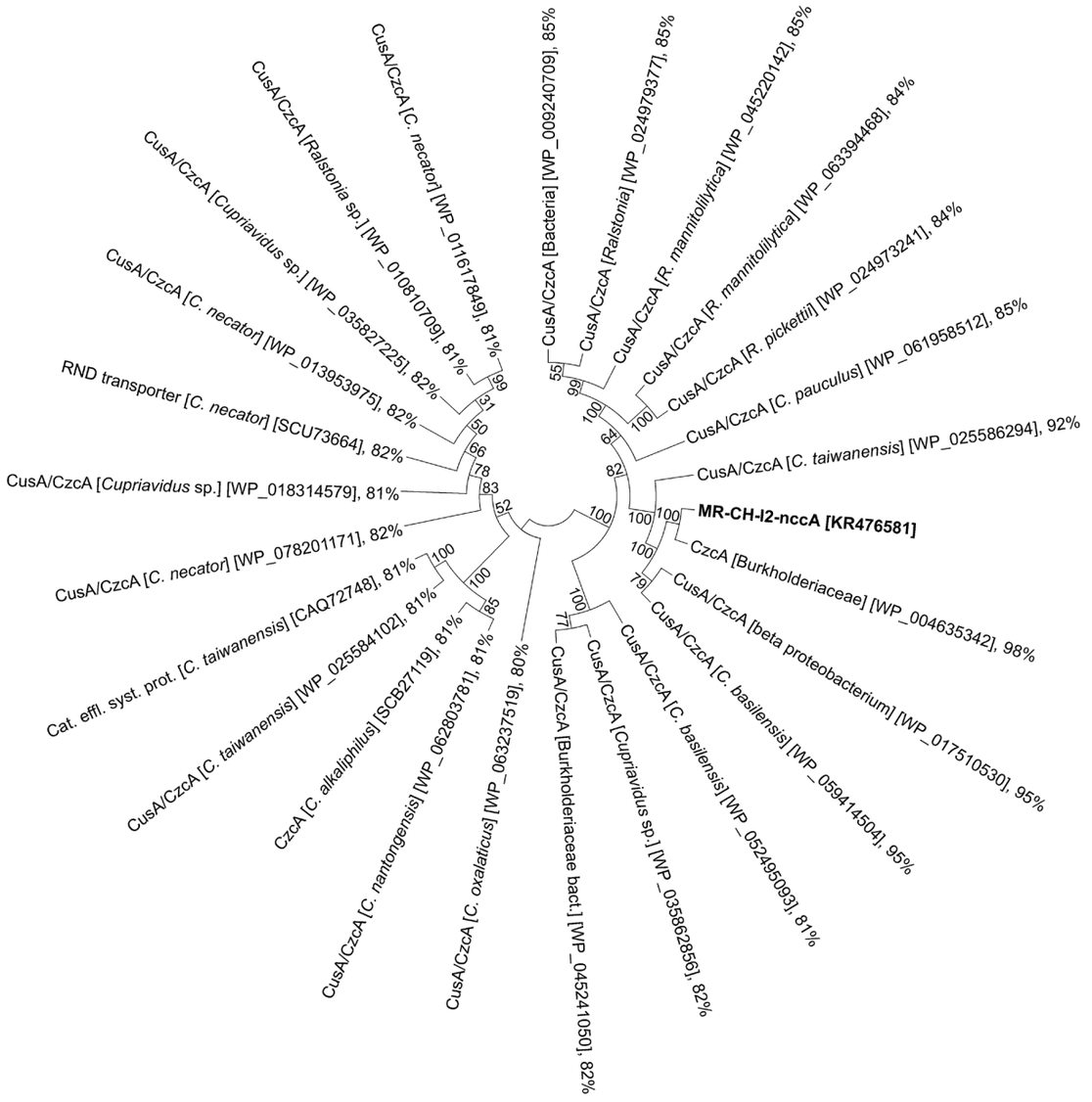

Fig. 5. Unrooted phylogenetic tree obtained by the maximum likelihood method with 100 bootstrap replications showing phylogeny of whole *ncc*A-like sequences based on 1,063 presented amino acid sites translated from DNA of the MR-CH-I2 isolate (in bold). Numbers in square brackets indicate the GenBank accession number and similarity to closest relative is shown after the clone designation. Sequences were aligned with ClustalW.

is 100% identical to WP_004635342.1 for "MULTISPECIES: cation efflux system protein CzcA [Burkholderiaceae]" over its full length. It is known that NCBI nonredundant RefSeq protein (WP_) can be annotated on large numbers of bacterial genomes that encode that identical protein (https://www.ncbi.nlm.nih.gov/protein/YP_001899332). The *czc* determinant encodes resistance to Cd^{2+}, Zn^{2+} and Co^{2+} by metal-dependent efflux driven by the proton motive force of the Czc-CBA complex (Nies, 1995). This system is the best-characterized CBA transporter from *C. metallidurans* CH34. Possession of this system makes a bacterium heavy metal resistant (Nies, 2003). Really, the isolate MR-CH-I2 showed relatively high level of resistance against nickel, cobalt, zinc and cooper and markedly higher level of resistance against cadmium. However, it did not carry resistance against any of investigated antibiotics and inhibited the growth of any of investigated bacteria (Remenár *et al.*, 2015).

Expression of *ncc*A-like heavy-metal resistance gene. Finally, RT-PCR experiments were done to search for expression of MR-CH-I2-nccA [KR476581] in the presence of different concentrations of five heavy metals (Ni = 250 μg/ml, Cd = 100 μg/ml, Zn = 50 μg/ml, Co = 25 μg/ml, Cu = 10 μg/ml). RNA was isolated either from these five heavy metal-induced MR-CH-I2 bacte-

Table II
Expression of MR-CH-I2-nccA [KR476581] gene after heavy metal additions to the medium.

Time (h)	Nickel[a]		Cadmium[a]		Cobalt[a]		Copper[a]		Zinc[a]	
	ΔΔCt	RQ	ΔΔCt	RQ	ΔΔCt	RQ	ΔΔCt	RQ	ΔΔCt	RQ
0	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00
2	−4.08	16.91	0.53	0.69	3.23	0.11	5.50	0.02	1.81	0.29
4	−0.17	1.13	6.32	0.01	5.18	0.03	6.61	0.01	3.17	0.11
6	−0.51	1.42	5.82	0.02	5.22	0.03	9.38	0.002	3.65	0.08
8	0.67	0.63	6.13	0.01	7.61	0.005	7.52	0.005	2.81	0.14

[a] Standardization of gene expression according to the house-keeping gene after heavy metal additions;
Ct – threshold cycle;
$\Delta\Delta Ct = \Delta Ct^1 (Ct_{nccA} - Ct_{nccA-Control}) - \Delta Ct^2 (Ct_{gdhA} - Ct_{gdhA-Control})$;
$RQ = 2^{-\Delta\Delta Ct}$;
*ncc*A – MR-CH-I2-nccA [KR476581] (nccA-like heavy-metal resistance gene) of MR-CH-I2 bacterium;
*gdh*A – the gene encodes glutamate dehydrogenase (house-keeping gene).

rial cells or from control sample (without heavy metal additions) in 0 h and 2, 4, 6 and 8 h after heavy metal additions. The expression gene pattern was normalised according to the reference gene *gdh*A. The results from RT-PCR analysis showed that only nickel after 2 h from its addition to the medium has significantly affected MR-CH-I2-nccA gene expression, it increased up to 16-times. The addition of remaining heavy metals did not significantly affect the MR-CH-I2-nccA gene expression (Table II). In fact, MR-CH-I2-nccA gene was identified as CzcA family heavy metal efflux pump [WP_004635342] from *R. picketii* 12J with 98% similarity (Fig. 5). However, this gene mediates inducible resistance to cobalt, zinck, and cadmium in *A. eutrophus* (Nies *et al.*, 1987; Nies, 1992; Kunito *et al.*, 1996). In addition, significant upregulation of *czc*A gene in *Pseudomonas aeruginosa* strain was found upon exposure only to low concentrations of zinc and cadmium for short duration of their influences on bacterium (Choudhary and Sar, 2016). Similarly, Abdelatey *et al.* (2011) have also confirmed the *ncc*A-like gene inductions by cobalt and cadmium additions to the medium in *Pseudomonas* sp. and *Bordetella* sp. strains isolated from heavy-metal contaminated soils. Thus, significant Ni-induction of MR-CH-I2-nccA gene is surprising because the function of the combined nickel-cobalt-cadmium resistance is mediated by the CzcCB2A-related NccCBA efflux system from *A. xylosoxidans* (Schmidt and Schlegel, 1994). But, in some occasions, Ni^{2+} and Co^{2+} are exported by the same CBA transporters as Zn^{2+} and Cd^{2+} (for example NccCBA from *A. xylosoxidans* 31A and CzcCBA from *C. metallidurans* CH34) (Schmidt and Schlegel, 1994; Legatzki *et al.*, 2003). This suggestion could explain MR-CH-I2-nccA gene induction only after nickel addition even because the natural environment of this bacterial strain was contaminated mainly by high nickel concentrations. These results suggest that we have obtained a new *R. picketii* strain MR-CH-I2 [MF102046] carrying MR-CH-I2-nccA [KR476581] heavy-metal resistance gene which is specific for particular contaminated sites, is cultivable, and has high pollutant-degradation activity.

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Literature

Abdelatey L.M., W.K.B. Khalil, T.H. Alit and K.F. Mahrous. 2011. Heavy metal resistance and gene expression analysis of metal resistance genes in Gram-positive and Gram-negative bacteria present in Egyptian soils. *J. Appl. Sci. Environ. Sanit.* 6: 201–211.

Adley C., J. Pembroke and M. Ryan. 2007. *Ralstonia pickettii* in environmental biotechnology potential and applications. *J. Appl. Microbiol.* 103: 754–764.

Bell C.W., V. Acosta-Martinez, N.E. McIntyre, S. Cox, D.T. Tissue and J.C. Zak. 2009. Linking microbial community structure and function to seasonal differences in soil moisture and temperature in a Chihuahuan desert grassland. *Microb. Ecol.* 58: 827–842.

Bogdanova E.S., I.A. Bass, L.S. Minakhin, M.A. Petrova, S.Z. Mindlin, A.A. Volodin, E.S. Kalyaeva, J.M. Tiedje, J.L. Hobman, N.L. Brown and V.G. Nikiforov. 1998. Horizontal spread of *mer* operons among Gram-positive bacteria in natural environments. *Microbiology* 144: 609–620.

Bohuš P. and J. Klinda. 2010. Environmentálna regionalizácia Slovenskej republiky. Bratislava: MŽP SR, Banská Bystrica: SAŽP, 2010, s. 9–21 (In Slovak).

Choudhary S. and P. Sar. 2016. Real-time PCR based analysis of metal resistance genes in metal resistant Pseudomonas aeruginosa strain J007. *J. Basic Microbiol.* 56: 688–697.

Coenye T., P. De Vos, J. Goris and P. Vandamme. 2003. Classification of *Ralstonia pickettii*-like isolates from the environment and clinical samples as *Ralstonia insidiosa*. *Int. J. Syst. Evol. Microbiol.* 53: 1075–1080.

Erbe J.L., K.B Taylor and L.M. Hall. 1995. Metalloregulation of the cyanobacterial *smt* locus: identification of SmtB binding sites and direct interaction with metals. *Nucleic Acids Res.* 23: 2472–2478.

Fett J., K. Konstantinidis, N. Isaacs, D. Long and T. Marsh. 2003. Microbial diversity and resistance to copper in metal-contaminated lake sediment. *Microbial Ecol.* 45: 191–202.

Gwrtac. 1997. Remediation of metals-contaminated soils and groundwater. *Tech. Evaluation. Rep.* 97:53.

Ji G. and S. Silver. 1992. Regulation and expression of the arsenic resistance operon from *Staphylococcus aureus* plasmid pI258. *J. Bacteriol.* 174: 3684–3694.

Johnson J.M. and G.M. Church. 1999. Alignment and structure prediction of divergent protein families: periplasmic and outer membrane proteins of bacterial efflux pumps. *J. Mol. Biol.* 287: 695–715.

Kaeberlein T., K. Lewis and S.S. Epstein. 2002. Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* 296: 1127–1129.

Karelová E., J. Harichová, T. Stojnev, D. Pangallo and P. Ferianc. 2011. The isolation of heavy-metal resistant culturable bacteria and resistance determinants from a heavy-metal-contaminated site. *Biologia* (*Bratislava*) 66: 18–26.

Kunito T., T. Kusano, H. Oyaizu, K. Senoo, S. Kanazawa and S. Matsumoto. 1996. Cloning and sequence analysis of *czc* genes in *Alcaligenes* sp. strain CT14. *Biosci. Biotechnol. Biochem.* 60: 699–704.

Lane D.J. 1991. 16S/23S rRNA sequencing, pp. 115–148. In: Stackebrandt E. and M. Goodfellow (eds). *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, New York.

Ławniczak Ł., A. Syguda, A. Borkowski, P. Cyplik, K. Marcinkowska, Ł. Wolko, T. Praczyk, Ł. Chrzanowski and J. Pernak. 2016. Influence of oligomeric herbicidal ionic liquids with MCPA and Dicamba anions on the community structure of autochthonic bacteria present in agricultural soil. *Sci. Total Environ.* 563–564: 247–255.

Legatzki A., S. Franke, S. Lucke, T. Hofmann, A. Anton, D. Neumann and D.H. Nies. 2003. First step towards a quantitative model describing Czc-mediated heavy metal resistance in *Ralstonia metallidurans*. *Biodegradation* 14: 153–168.

Liu H.Y., A. Probs and B. Liao. 2005. Metal contamination of soils and crops affected by the Chenzhou lead/zinc mine spill (Hunan, China). *Sci. Total Environ.* 339: 153–166.

Mergeay M., S. Monchy, T. Vallaeys, V. Auquier, A. Benotmane, P. Bertin, S. Taghavi, J. Dunn, D. Van der Lelie and R. Wattiez. 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. *FEMS Microbiol. Rev.* 27: 385–410.

Mobley H.L., C.M. Chen, S. Silver and B.P. Rosen. 1983. Cloning and expression of R-factor mediated arsenate resistance in *Escherichia coli*. *Mol. Gen. Genet.* 191: 421–426.

Nascimento A.M.A. and E. Chartone-Souza. 2003. Operon *mer*: bacterial resistance to mercury and potential for bioremediation of contaminated environments. *Genet. Mol. Res.* 2: 92–101.

Nies D., M. Mergeay, B. Friedrich and H.G. Schlegel. 1987. Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus* CH34. *J. Bacteriol.* 169: 4865–4868.

Nies D.H. 1992. Resistance to cadmium, cobalt, zinc and nickel in microbes. Plasmid 27: 17–28.

Nies D.H. 1995. The cobalt, zinc, and cadmium efflux system CzcABC from *Alcaligenes eutrophus* functions as a cation-proton-antiporter in *Escherichia coli*. *J. Bacteriol.* 177: 2707–2712.

Nies D.H. 1999. Microbial heavy metal resistance. *Appl. Microbiol. Biotechnol.* 51: 730–750.

Nies D.H. 2003. Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol. Rev.* 27: 313–339.

Nováková R., R. Knirschová, M. Farkašovský, Ľ. Fecková, A. Reháková, E. Mingyar and J. Kormanec. 2013. The gene cluster *aur1* for the angucycline antibiotic auricin is located on a large linear plasmid pSA3239 in *Streptomyces aureofaciens* CCM 3239. *FEMS Microbiol. Lett.* 342: 130–137.

Panagos P., M. Van Liedekerke, Y. Yigini and L. Montanarella. 2013. Contaminated sites in Europe: Review of the current situation based on data collected through a European network. *J. Environ.* Public Health. 2013: 11 pp. ID 158764. http://dx.doi.org/10.1155/2013/158764

Paulsen I.T., J.H. Park, P.S. Choi and M.H.J. Saier. 1997. A family of Gram-negative bacterial outer membrane factors that function in the export of proteins, carbohydrates, drugs and heavy metals from Gram-negative bacteria. *FEMS Microbiol. Lett.* 156: 1–8.

Remenár, M., E. Karelová, J. Harichová, M. Zámocký, A. Kamlárová and P. Ferianc. 2015. Isolation of previously uncultivable bacteria from a nickel contaminated soil using a diffusion-chamber-based approach. *Appl. Soil Ecol.* 95: 115–127.

Rosenstein R., A. Peschel, B. Wieland and F. Götz. 1992. Expression and regulation of the antimonite, arsenite, and arsenate resistance operon of *Staphylococcus xylosus* plasmid pSX267. *J. Bacteriol.* 174: 3676–3683.

Saier M.H.J., R. Tam, A. Reizer and J. Reizer. 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. *Mol. Microbiol.* 11: 841–847.

Saltikov C.W. and B.H. Olson. 2002. Homology of *Escherichia coli* R773 *ars*A, *ars*B, and *ars*C genes in arsenic-resistant bacteria isolated from raw sewage and arsenic-enriched creek waters. *Appl. Environ. Microbiol.* 68:280–288.

Salvador M., G. Carolina and E. Jose. 2007. Novel nickel resistance genes from the rhizosphere metagenome of plants adapted to acid mine drainage. *Appl. Environ. Microbiol.* 73: 6001–6011.

Schmidt T. and H.G. Schlegel. 1994. Combined nickel-cobaltcadmium resistance encoded by the ncc locus of *Alcaligenes xylosoxidans* 31A. *J. Bacteriol.* 176: 7045–7054.

Shiowatana J., R.G. McLaren, N. Chanmekha and A. Samphao. 2001. Fractionation of arsenic in soil by a continuous-flow sequential extraction method. *J. Environ. Qual.* 30: 1940–1949.

Silver S. and L.T. Phung. 1996. Bacterial heavy metal resistance: New surprises. *Ann. Rev. Microbiol.* 50: 753–789.

Stackebrandt E., R.G.E. Murray and H.G. Trüper. 1988. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the „Purple Bacteria and Their Relatives". *Int. J. Syst. Bacteriol.* 38: 321–325.

Sydow M., M. Owsianiak, Z. Szczepaniak, G. Framski, B.F. Smets, Ł. Ławniczak, P. Lisiecki, A. Szulc, P. Cyplik and Ł. Chrzanowski. 2016. Evaluating robustness of a diesel-degrading bacterial consortium isolated from contaminated soil. *N. Biotechnol.* 33: 852–859.

Sydow M., L. Chrzanowski, N. Cedergreen and M. Owsianiak. 2017. Limitations of experiments performed in artificially made OECD standard soils for predicting cadmium, lead and zinc toxicity towards organisms living in natural soils. *J. Environ. Manage.* 198(Pt 2): 32–40.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nie and S. Kumar. 2011. "MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods". *Mol. Biol. Evol.* 28: 2731–2739.

Tchounwou P.B., C.G. Yedjou, A.K. Patlolla and D.J. Sutton. 2012. Heavy metals toxicity and the environment. *EXS* 101: 133–164.

Thiyagarajan V., S. Lau, M. Tsoi, W. Zhang and P.Y. Qian. 2010. Monitoring bacterial biodiversity in surface sediment using terminal restriction fragment length polymorphism analysis (T-RFLP): Application to coastal environment, pp. 151–163. In: Ishimatsu A. and H.-J. Lie (eds). *Coastal environmental and ecosystem issues of the East China sea*. TERRAPUB & Nagasaki University.

Tóth G., T. Hermann, M.R. Da Silva and L. Montanarella. 2016. Heavy metals in agricultural soils of the European Union with implica-tions for food safety. *Environ. Int.* 88: 299–309.

Tseng T.-T., K.S. Gratwick, J. Kollman, D. Park, D.H. Nies, A. Goffeau and M.H.J. Saier. 1999. The RND superfamily: an

ancient, ubiquitous and diverse family that includes human disease and development proteins. *J. Mol. Microbiol. Biotechnol.* 1: 107–125.
Wuana R.A. and F.E. Okieimen. 2011. Heavy metals in contaminated soils: A review of sources, chemistry, risks and best available strategies for remediation. *ISRN Ecology* 2011: 20 pp. ID 402647. doi:10.5402/2011/402647
Yamamoto S. and S. Harayama. 1995. PCR amplification and direct sequencing of *gyr*B genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl. Environ. Microbiol.* 61: 1104–1109.
Zgurskaya H.I. and H. Nikaido. 1999a. Bypassing the periplasm: Reconstitution of the AcrAB multidrug efflux pump of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 96: 7190–7195.
Zgurskaya H.I. and H. Nikaido. 1999b. AcrA is a highly asymmetric protein capable of spanning the periplasm. *J. Mol. Biol.* 285: 409–420.
Zgurskaya H.I. and H. Nikaido. 2000a. Multidrug resistance mechanisms: drug efflux across two membranes. *Mol. Microbiol.* 37: 219–225.
Zgurskaya H.I. and H. Nikaido. 2000b. Cross-linked complex between oligomeric periplasmic lipoprotein AcrA and the inner-membrane-associated multidrug efflux pump AcrB from *Escherichia coli*. *J. Bacteriol.* 182: 4264–4267.
"*Ralstonia pickettii*" JGI Genome Portal – Home. Web. 2010. http://genome.jgi-psf.org/ralpd/ralpd.home.html.
"HAMAP: *Ralstonia pickettii* (strain 12D) complete proteome." ExPASy Proteomics Server. Swiss Institute for Bioinformatics. Web. 2010. http://www.expasy.ch/sprot/hamap/RALP1.html.
"HAMAP: *Ralstonia pickettii* (strain 12J) complete proteome." ExPASy Proteomics Server. Swiss Institute for Bioinformatics. Web. 2010. http://expasy.org/sprot/hamap/RALPJ.html.
https://www.ncbi.nlm.nih.gov/protein/YP_001899332

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ORIGINAL PAPER

Distribution of Cell Envelope Proteinases Genes among Polish Strains of *Lactobacillus helveticus*

KATARZYNA W. SKRZYPCZAK[1]*, WALDEMAR Z. GUSTAW[1] and ADAM D. WAŚKO[2]

[1]Department of Fruits, Vegetables and Mushrooms Technology, Faculty of Food Science and Biotechnology, University of Life Sciences in Lublin
[2]Department of Biotechnology, Human Nutrition and Food Commodity Science, Faculty of Food Science and Biotechnology, University of Life Sciences in Lublin

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Abstract

Most of the lactic acid bacteria (LAB) are able to grow in milk mainly due to the activity of a complex and well-developed proteolytic system. Cell envelope-associated proteinases (CEPs) begin casein hydrolysis and allow for releasing the peptides, enclosed in the structure of native milk proteins that are essential for growth of *Lactobacillus helveticus.* The biodiversity of genes encoding CEPs among *L. helveticus* strains can have an effect on some technological parameters such as acid production, bacterial growth rate in milk as well as liberation of biologically active peptides. The study reveals significant differences in the presence of various variants of CEPs encoding genes among ten novel Polish strains and indicates the intraspecific diversity exhibited by *L. helveticus*. In terms of distribution of CEPs genes, four different genetic profiles were found among the microorganisms analyzed. Furthermore, the strains exhibited also various levels of proteolytic activity. Molecular analysis revealed that *prt*H3 is the most abundant CEPs-encoding gene among the strains investigated. The results indicate also that ecological niche and environmental conditions might affect proteolytic properties of *L. helveticus* strains. The greatest variety in terms of quantity of the detected CEP encoding genes was noticed in *L. helveticus* 141, T105 and T104 strains. In these strains, the combination of three nucleotide gene sequences (*prt*H/*prt*H2/*prt*H3) was identified. Interestingly, T104 and T105 exhibited the highest proteolytic activity and also the fastest dynamic of milk acidification among the tested strains of *L. helveticus*.

Key words: *Lactobacillus helveticus*, cell envelope proteinases (CEPs), proteolytic activity

Introduction

Lactobacillus helveticus is recognized as the most nutritionally fastidious lactic acid bacteria (LAB) that are unable to synthesize some of the essential for its growth amino acids. A complex proteolytic system enables the bacteria to grow in milk mainly due to overcoming their amino acids auxotrophies and providing available source of nitrogen (Genay *et al*., 2009). Generally, the proteolytic system consists of three components: cell-envelope proteinases (CEPs) that hydrolyze caseins into oligopeptides, transport system that transfer oligopeptides across the membrane inside the bacterial cell, and finally intracellular peptidases that generate free intracellular amino acids (Savijoki *et al.*, 2006; Sadat-Mekmene *et al.*, 2011b).

L. helveticus strains are used as starter cultures mainly due to their high tolerance to low pH, the rate of milk acidification and acid curd formation (Nielsen *et al.,* 2009). Moreover, the bacteria strains may also hydrolyze hydrophobic peptides such as peptide β-CN (193–209) and therefore significantly reduce the bitter taste of cheese (Sadat-Mekmene *et al.,* 2011a; 2011b).

Cell wall-associated proteases play a crucial role in cheese maturation because contribute to release of hydrophobic peptides and create stretching properties of cheeses (Oommen *et al.*, 2002; Richoux *et al.*, 2009). In addition, the products obtained with proteolytic activities of *L. helveticus* exhibit a wide range of health-promoting effects mainly due to bioactive peptides (Griffiths and Tellez, 2013). However, a huge biodiversity in terms of CEPs has been noticed among different strains of *L. helveticus.* The individual strain might exhibit from 1 to 4 various types of cell wall-associated proteases (Sadat-Mekmene *et al.*, 2013). This is also related to different combinations of CEPs-encoding genes that probably affect enzymes activity and constitute an important aspect for applications the

* Corresponding author: K.W. Skrzypczak, Department of Fruits, Vegetables and Mushrooms Technology, Faculty of Food Science and Biotechnology, University of Life Sciences in Lublin, Poland; **e-mail: katarzyna.skrzypczak@up.lublin.pl**

individual strains in dairy industry (Broadbent *et al.*, 2011; Sadat-Mekmene *et al.*, 2011b). Nonetheless, some aspects referring to the CEPs properties in *L. helveticus* strains are still not fully explained (Savijoki *et al.*, 2006; Sadat-Mekmene *et al.*, 2011b). Therefore, the objectives of this investigation were to evaluate the distribution of genes encoding cell-envelope proteinases and to determine the proteolytic activities of novel Polish strains of *L. helveticus*. Hence, the research results are of importance in the potential application of *L. helveticus* strains in local dairy industry.

Experimental

Materials and Methods

Bacterial strains and growth conditions. Ten strains of lactic acid bacteria (80, 141, T15, T80, T103, T104, T105, T159, T199, B734) were isolated from fermented Polish milk products and kindly provided by Prof. Łucja Łaniewska-Trokenheim (University of Warmia and Mazury in Olsztyn, Poland). The microorganisms have not yet been used industrially. The strains were previously identified by 16S rRNA sequence analysis in our laboratory.

L. helveticus K1 strain isolated from Canadian dairy product and obtained from the Division of Food Science Institute of Animal Reproduction and Food Research of The Polish Academy of Science (Olsztyn, Poland) was also included in the study. Moreover, *L. helveticus* DSMZ 20075 (DSMZ, Braunschweig, Germany) was used as a reference strain, while *Lactobacillus rhamnosus* E/N (BIOMED-LUBLIN WSiS S.A, Lublin, Poland) was a negative control.

All strains were maintained in 15% glycerol stock and stored at –80°C. Prior to the experiments, each bacterial strain was transferred into fresh sterile medium cultured (2%v/v) in De Man, Rogosa and Sharpe broth (BTL, Łódź, Poland) supplemented with L-cysteine (0.5 g/l) and incubated (42°C/16 h) under anaerobic conditions (Waśko *et al.*, 2014).

Extraction of DNA and the species-specific PCR. Total cellular DNA was isolated from overnight strains cultures by Genomic Mini AX Bacteria Spin (A&A Biotechnology, Poland). The reaction of amplification of housekeeping genes of *L. helveticus* was performed with using specific primers according to Fortina *et al.* (2001). The multiplex PCR reaction was conducted using the LabCycler (SensoQuest, Göttingen, Germany). The obtained amplification products were electrophoresed in 1% agarose gel with addition of 0.25% Midori Green DNA Stain (Nippon Genetics Europe, Dueren, Germany). The electrophoresis was conducted in TBE buffer for 1.5 h at 60 V, visualized under UV light using GelDoc (Bio-Rad, USA) and further analyzed in Quantity One (Bio-Rad, USA).

Detection the genes encoding CEPs. The reactions of amplification of *prt*H, *prt*H2, *prt*H3 and *prt*H4 were performed according to Broadbent *et al.* (2011) with primers listed in Table I. Each reaction mixture (25 µl) contained 100 ng of DNA, 12.5 µl DreamTaq Green PCR Master Mix (2X) (Life Technologies Sp. z o.o., Warsaw, Poland), 20 pmol of each primer and nuclease-free water. The PCR reaction steps included: 4 min denaturation at 95°C followed by 30 cycles consisting on three steps (95°C for 30 s, 58°C for 30 s and 72°C for 30 s). The final extension was conducted 10 min at 72°C. The obtained PCR products were directly subjected to electrophoresis as it has been described above.

DNA sequences analyses. The selected PCR products obtained in the study were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the capillary sequencing system, 3730 Genetic Analyzer (Applied Biosystems). The consensus sequences from the alignments were analyzed (using BLAST, ClustalW and ClustalOmega) and compared to other sequences available in GenBank database. The nucleotide sequences of cell envelope-associated proteinase genes found in this study were deposited in the GenBank database with the accession numbers: KT285174, KT285175, KT285176, KT285177, KT285180 and KT285181.

Table I
Sequences of primers used in reaction of amplifications of the fragments of CEPs genes.

Gen	Primer	Sequence (5'→3')	Tm [°C]	Source
prtH	PrtH-for-1 PrtH-rev-1	GGTACTTCAATGGCTTCTCC GATGCGCCATCAATCTTCTT	51.8 49.7	Genay *et al.*, 2009; Lozo *et al.*, 2011
prtH2	prtH2f prtH2r	AAGCAAAGGATGTTGTTCCAAGTAAGCCA CTCTCTTCCTTCTTACCAGTTGATGATTGAACT	58.7 60.7	Smeianov *et al.*, 2007
prtH3	prtH3f prtH3r	GATGATCAAGCAGATGTAAAACCGGCAGAAG ATTTACTGAAGAATTAGTCAAATGACCTGTTGTCGG	61.7 61.0	Broadbent *et al.*, 2011
prtH4	prtH4f prtH4r	CTGAAGCAGCAACTAATGATCCTGG TGGATTAGGATCCGTTCTGGTTGTCAG	57.7 59.7	Broadbent *et al.*, 2011

Proteolytic activity assay. In order to determine the proteolytic activity of the analyzed microorganisms, 10 ml of sterile MRS broth (BTL, Łódź, Poland) was inoculated by 1% (v/v) of overnight cultures of *L. helveticus* strains and incubated 18 h at 42°C under anaerobic conditions. Then, the bacterial cells were collected by centrifugation at 10 000 g for 10 min at 4°C (Eppendorf Centrifuge 5415R, Eppendorf Hamburg, Germany). The pellets were washed twice with phosphate buffer (0.1 M, pH 7.0) and using the same buffer resuspended to the original volume. The obtained bacterial suspensions were incorporated as 1% (v/v) inoculum into 10% (w/v) reconstituted and sterilized (115°C/15 min) skim milk (OSM Krasnystaw, Krasnystaw, Poland). All samples were mixed by vortexing and incubated (42°C/12 h) under anaerobic conditions. Uninoculated milk samples were used as a control. Proteolytic activities of milk-grown cultures were determined spectrophotometrically (Smartspec Plus, BioRad, Hercules, CA, USA) according to Savoy de Giori and Herbert (2001).

A statistical analysis was performed using a statistical program Statistica 13.1 (StatSoft, Tulsa, USA). The proteolytic activities exhibited by the strains analyzed were presented as mean value with standard deviations. The Tukey's HSD test was used to estimate the significant differences between mean values. The obtained results were compared on the basis of significance level set at $p < 0.05$.

Acidifying activity. The overnight cell cultures were harvested by centrifugation (8 000 × g/10 min/4°C), washed twice with sodium phosphate buffer (50 mM, pH 7.0) and resuspended in the same buffer to the original sample volume. The obtained bacterial cell suspensions ($OD_{600} = 0.7$) were used to inoculate samples of 13% (w/v) regenerated skim milk (RSM) (OSM Krasnystaw, Poland), which had been pasteurized in water bath (80°C/30 min) and cooled down to room temperature before inoculation.

During the whole time of fermentation (36 h/42°C), the value of pH was measured (pH meter Hanna Instruments HI221) every 6 h. The measurements were done in triplicate (in sterile conditions). The dynamics of milk acidification by individual *L. helveticus* strain has been expressed as a difference (ΔpH) between measurements that were done in 6-hour intervals during all fermentation time. Strains, which were able to reduce the pH value of RSM more than one unit within the first six hours of incubation, were considered as fast acidifying.

Results

The results of multiplex PCR indicated the presence of 500, 700, and 900 bp bands (Fig. 1). The presence of these three products was confirmed in all tested strains.

To determine the distribution of CEPs-encoding genes among the tested strains of *L. helveticus*, three reactions of amplification of the nucleotides sequences (*prt*H, *prt*H2 and *prt*H3) were applied. The results indicated that presence of CEPs-encoding genes and their combination varied among the microorganisms tested. The results (Fig. 2A) demonstrated that sequence of *prt*H was presented in four *L. helveticus* strains (T104, T105, 141 and B734). Among the bacteria tested, the *prt*H2 proved to be more common CEPs-encoding sequence than *prt*H (Fig. 2B), while *prt*H3 was the most widespread gene and was detected in all strains (Fig. 2C). However, any product of amplification *prt*H4 was obtained and thus, the presence of this gene has not been confirmed in any of the tested *L. helveticus* strains.

Depending on the variant of detected CEPs-encoding sequences, the analyzed strains were distinguished into four genetic profiles. The strains exhibiting presence of sequences *prt*H, *prt*H2 and *prt*H3 (T104, T105 and 141) were qualified to profile I (Table II). In profile II, the strains exhibiting the presence of *prt*H/*prt*H3 genes combination were grouped, while in the strains comprising the profile III, the genes *prt*H2 and *prt*H3 were detected. Profile IV was represented by strains, in which only one product of amplification (*prt*H3) was observed. The greatest diversity in terms of the number of identified CEPs-encoding nucleotide sequences was shown for strains 141, T104 and T105.

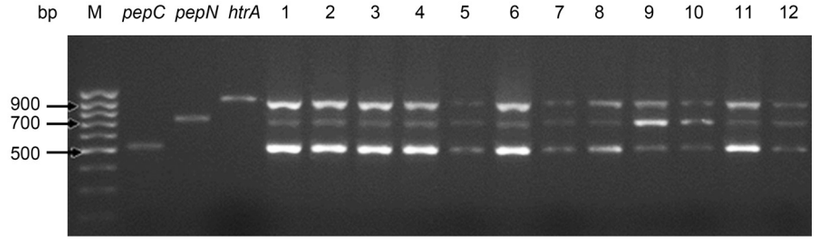

Fig. 1. Agarose gel electrophoresis of Multiplex PCR products obtained for *Lactobacillus helvetisus* strains: 1 – 80; 2 – T104; 3 – T105; 4 – T159; 5 – 14; 6 – B734; 7 – T103; 8 – T15; 9 – T199; 10 – T80; 11 – K1; 12 – DSMZ 20075; M – DNA molecular marker 100 bp.

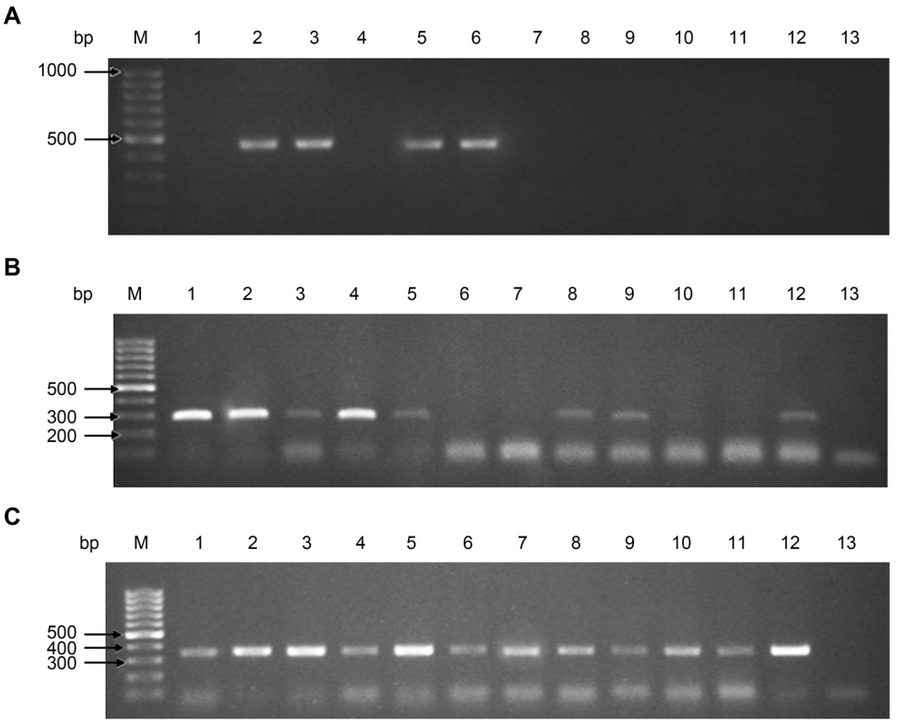

Fig. 2. Results of amplification genes encoding CEPs: *prt*H (A); *prt*H2 (B); *prt*H3 (C) in *Lactobacillus helvetisus* strains: Line: 1 – 80; 2 – T104; 3 – T105; 4 – T159; 5 – 141; 6 – B734; 7 – T103; 8 – T15; 9 – T199; 10 – T80; 11 – K1; 12 – DSMZ 20075; line 13: *Lactobacillus rhamnosus* E/N; M – DNA molecular marker.

The choice of amplification products for bioinformatics analysis was based on the results of proteolytic activity assay and distribution of the CEPs genes among the tested strains. Therefore, to further nucleotide sequence analysis were subjected all amplified *prt*H products and also amplicons of *prt*H3, which were detected in strains T80, T105, T104, 141 and B734.

A multiple sequence alignment (Fig. 3) of the nucleotide sequences of *prt*H exhibited slight differences between the strains tested and *L. helveticus* CRZN32

Table II
The proteolytic activity of *L. helveticus* strains.

The bacterial strain	Proteolytic activity [mM of released α-aminoacids/l]	Profiles of amplification products of CEPs
L. helveticus T104	$87.06^{c} \pm 0.21$	I (*prt*H/*prt*H2/*prt*H3)
L. helveticus T105	$114.72^{a} \pm 0.64$	
L. helveticus 141	$57.67^{d} \pm 0.54$	
L. helveticus B734	$58.78^{d} \pm 0.52$	II (*prt*H/*prt*H3)
L. helveticus 80	$37.78^{h} \pm 0.68$	III (*prt*H2/*prt*H3)
L. helveticus T159	$42.67^{e} \pm 0.14$	
L. helveticus T15	$40.61^{fg} \pm 0.48$	
L. helveticus T199	$40.61^{fg} \pm 0.34$	
L. helveticus DSMZ 20075	$96.94^{b} \pm 1.1$	
L. helveticus T103	$41.33^{ef} \pm 0.36$	IV (*prt*H3)
L. helveticus T80	$39.78^{fg} \pm 0.28$	
L. helveticus K1	$40.11^{fg} \pm 0.42$	
L. rhamnosus E/N	$39.11^{gh} \pm 0.44$	–

The means (data are expressed as the mean ± standard deviations (SD), n = 3) in the same column, followed by different lower case letters, denote that they are significantly different ($p < 0.05$)

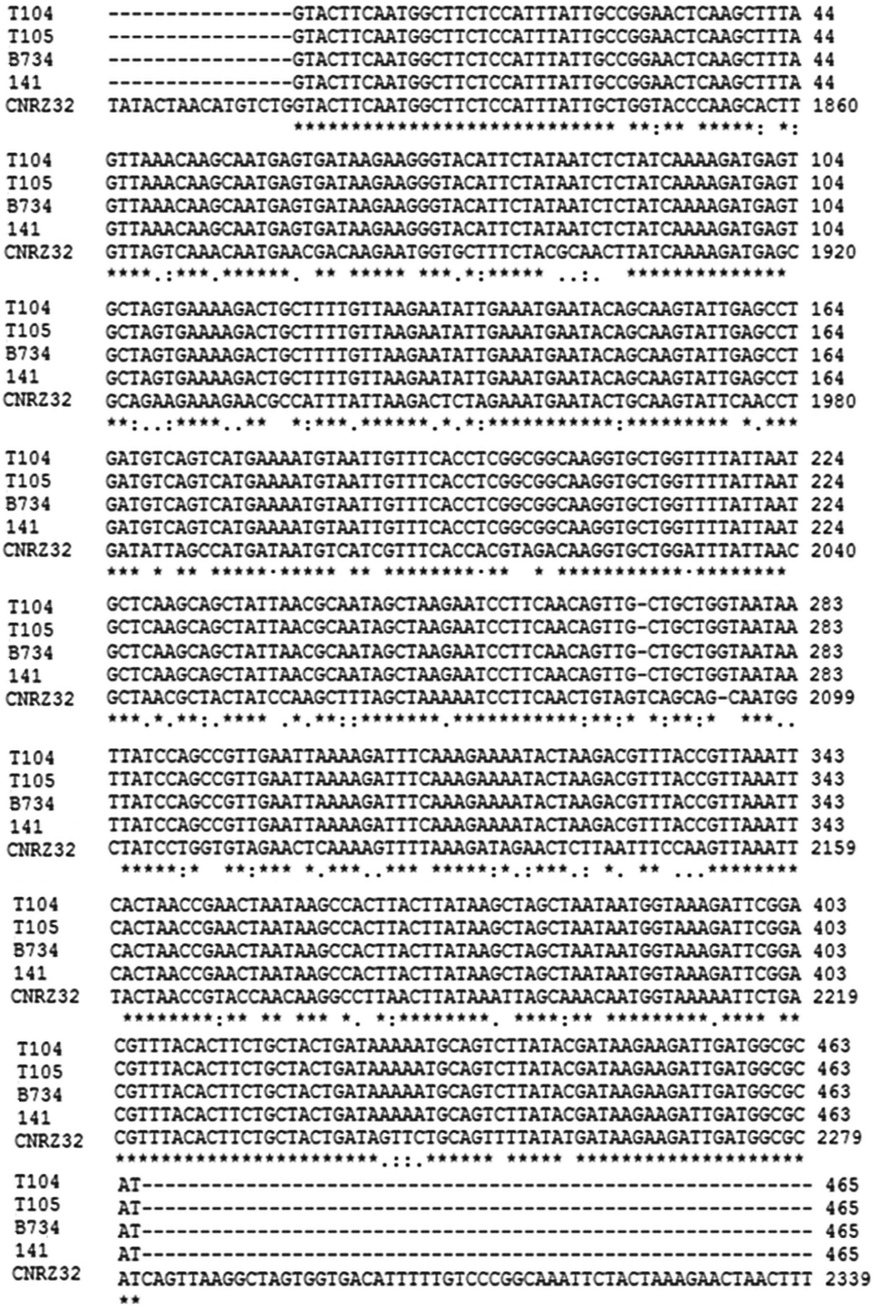

Fig. 3. Sequence alignment for *prt*H of chosen strains and *Lactobac illus helveticus* CRZN32 (no. AF133727). Stars indicate residues that are similar in all sequences.

(accession no. AF133727). Whereas, the analysis of phylogenetic tree (Fig. 4) demonstrated that the analyzed *prt*H3 gene sequences of T105 and T80 exhibited a higher similarity to the reference gene (accession no. HQ602769) than strains T104, 141 and B734.

The proteolytic activity was variable among the strains tested (Table II), while the strongest activity was exhibited by *L. helveticus* T105, a comparable value was recorded for the reference strain (DSMZ 20075). The lowest value of the measured parameter was noted for *L. helveticus* 80. Acidification of reconstructed skim milk (RSM) seems to be strain-dependent (Table III).

Most of the analyzed strains exhibited the strongest acidification activity during first 6 h of incubation. Moreover, *L. helveticus* T104 and T105 were able to reduce pH of RSM within the first 6 h of fermentation to more than one unit. Therefore, T104 and T105 were considered as the fast acidifying strains.

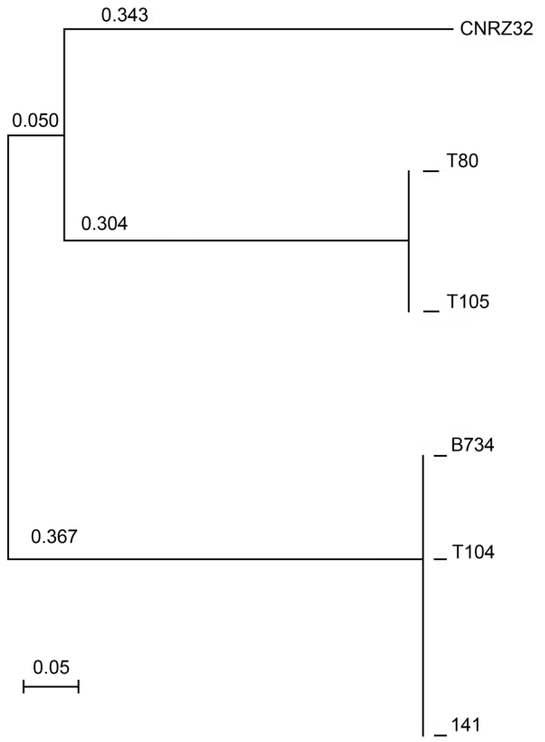

Fig. 4. Phylogenetic tree of *prt*H3 gene sequences of analyzed Polish *L. helveticus* strains and *L. helveticus* CNRZ32 (no. HQ602769.1).

Discussion

L. helveticus is an essential component of starter cultures in manufacture of ripened cheeses, especially Italian and Swiss-type. These microorganisms contribute to biochemical changes that influence the texture formation, development of sensory and organoleptic properties of final products (Soeryapranata *et al.*, 2007; Widyastuti *et al.*, 2014). However, a wide variability occurs among *L. helveticus* strains and also difficulties in distinguishing *L. helveticus* and closely related species e.g. *L. acidophilus* and *L. delbrueckii* (Rong *et al.*, 2015).

The multiplex PCR based on the amplification of the genes of stable and essential proteins for *L. helveticus* metabolism (housekeeping genes) is used for identification or confirmation of taxonomic affiliation. This method is used for rapid and unambiguous identification of *L. helveticus* strains (Fortina *et al.*, 2011; Rong *et al.*, 2015). In this study, the identified multiplex PCR products corresponded to the results obtained by Fortina *et al.* (2011), who described these products as the genes encoding: a trypsin-like serine protease (*htrA*), and aminopeptidases C (*pepC*) and N (*pepN*). Similar amplification products were identified in probiotic strain *L. helveticus* NS8, which was isolated from a traditional Mongolian fermented milk beverage (kumys) (Rong *et al.*, 2015).

It was revealed that *L. helveticus* exhibits intraspecific diversity and even biotypes isolated from the same niche are greatly various and many traits of the bacteria are strain-dependent (Griffiths and Tellez, 2013; Gatti *et al.*, 2014). Similar observations were noted in our study. The analyzed strains exhibited various proteolytic activity levels as well as diverse dynamic of milk acidification. These properties are one of the most important criteria determining the possibility of commercial applications of LAB in dairy industry (Ravyts *et al.*, 2012).

Many species belonging to the lactic acid bacteria possess only one type of cell envelope proteinases; therefore, *L. helveticus* exhibiting from one to four

Table III
Dynamics of decrease of skim milk pH value during fermentation conducted by *L. helveticus* strains.

L. helveticus strain	ΔpH*					
	6 h	12 h	18 h	24 h	30 h	36 h
T104	1.31 ± 0.01	1.35 ± 0.01	0.48 ± 0.01	0.03 ± 0.01	0.08 ± 0.01	0
T105	1.79 ± 0.01	1.23 ± 0.02	0.24 ± 0.02	0.05 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
141	0.8 ± 0.01	0.62 ± 0.01	0.76 ± 0.01	0.07 ± 0.02	0.22 ± 0.01	0.13 ± 0.01
B734	0.86 ± 0.02	0.24 ± 0.01	0.75 ± 0.01	0.06 ± 0.01	0.5 ± 0.02	0
80	0.8 ± 0.01	0.21 ± 0.01	0.78 ± 0.01	0.09 ± 0.01	0.49 ± 0.01	0.17 ± 0.01
T159	0.90 ± 0.01	0.15 ± 0.03	0.65 ± 0.02	0.22 ± 0.02	0.53 ± 0.01	0.23 ± 0.01
T15	0.74 ± 0.01	0.16 ± 0.02	1.05 ± 0.01	0.09 ± 0.01	0.51 ± 0.01	0
T199	0.85 ± 0.01	0.23 ± 0.02	0.82 ± 0.01	0.04 ± 0.01	0.49 ± 0.01	0.28 ± 0.01
DSMZ 20075	0.89 ± 0.02	0.66 ± 0.03	0.31 ± 0.01	0.66 ± 0.01	0.22 ± 0.01	0
T103	0.76 ± 0.01	0.33 ± 0.02	1.06 ± 0.02	0.20 ± 0.01	0.25 ± 0.01	0
T80	0.93 ± 0.01	0.89 ± 0.01	0.18 ± 0.01	0.54 ± 0.01	0.07 ± 0.01	0.21 ± 0.01
K1	0.82 ± 0.03	0.24 ± 0.02	0.76 ± 0.01	0.14 ± 0.01	0.56 ± 0.03	0.26 ± 0.01

* Data are expressed as the mean ± standard deviations (SD) (n = 3) of differences in pH values between measurements that were made after every 6 h of fermentation. The initial pH ranged from 6.59 to 6.62

CEPs appears to be a unique microorganism among all LAB (Genay *et al*., 2009; Broadbent *et al.,* 2011; Sadat-Mekmene *et al.,* 2013; Nejati *et al.,* 2016).

Due to a varied number of CEPs-encoding genes that were detected in the study, the *L. helveticus* strains were divided into four genetic profiles. Interestingly, T104 and T105 showed the highest proteolytic activity among the tested strains and the most diverse distribution of genes encoding CEPs. Beyond that, both strains exhibited also the fastest dynamics of decrease of milk pH value during fermentation process. However, the results obtained by Sadat-Mekmene *et al.* (2011a) indicated that the ability to acidify milk is a strain-dependent characteristic of *L. helveticus,* but no correlation has been confirmed between the rate of lowering the pH of milk and the number of different CEPs present in the strains. Nevertheless, the issue concerning the correlation between proteolytic activity level, the number of different CEPs and the genes encoding these enzymes still seems to be essential subject of considerations.

It has been demonstrated that *L. helveticus* CM4 characterized by a very high proteolytic activity exhibits the presence of three different CEPs-encoding genes (Wakai and Yamamoto, 2012). Similar findings have been also recorded for strains T104 and T105 in this study.

It was suggested that variations of cell envelope proteinase might demonstrate some differences in terms of affinity and specificity to particular casein fractions (Kunji *et al.*, 1996). In analysis of *L. helveticus* BGRA43 (Lozo *et al.*, 2011) the presence of only one CEP-encoding gene (*prt*H) was confirmed. Despite of this, the strain showed a high efficiency of the proteolytic system and was able to conduct a complete hydrolysis of αs1-, β- and κ-casein. While, other investigation of *L. helveticus* strains derived from different niches indicated that these strains were able to perform fast β-casein hydrolysis, regardless of whether they possessed one (PrtH2) or both variants of enzymes (PrtH and PrtH2) (Sadat-Mekmene *et al.,* 2011a). Whereas, α_{s1}-casein was much slower hydrolyzed by strains with only one CEP. It might be concluded that affinity and specificity to different casein fractions exhibited by CEPs of *L. helveticus* affect the composition and functional properties of the hydrolyzates received (Oberg *et al.*, 2002). Therefore, the analysis of distribution the CEP-encoding genes in *L. helveticus* strains, as a one of the selection traits for determination of the starter culture composition, seems to be justified.

The genes responsible for metabolism of peptidases and amino acids are highly conserved through the species, whereas sequences encoding CEPs are widely diverse (Broadbent *et al.* 2011).

Genay *et al.* (2009) have confirmed that the distribution of *prt*H and *prt*H2 is strain-dependent. In the study, *prt*H2 was detected in all of the 29 tested strains, whereas *prt*H was identified in 18 of them. Also in study presented by Nejati *et al.* (2016) *prt*H2 was identified in all of eight investigated *L. helveticus* strains, while presence of *prt*H has been confirmed in the four of them. In contrast, studies conducted by Miyamoto *et al.* (2015) in order to determine the distribution of CEPs genes in *L. helveticus* strains isolated from Airag (traditional Mongolian fermented milk product), showed that amplification products of *prt*H were present in six of seven strains tested, while *prt*H2 was identified only in two of them.

The results obtained for Polish strains of *L. helveticus* revealed that *prt*H2 occurred more frequently than *prt*H, which was detected only in four of twelve strains tested. Interestingly, *prt*H was identified in strains that exhibited presence of at least one another sequence encoding CEPs. Similar findings were reported by Broadbent *et al.* (2011), who noticed that *prt*H often occurred in combination with other gene encoding CEPs.

Analyzing results of all performed PCR reactions, it was noticed that not *prt*H2, but *prt*H3 was the most widespread gene among the strains analyzed. For some strains *prt*H3 was the only identified sequence of the CEP gene. These findings are in accordance with Broadbent *et al.* (2011), who also revealed intraspecific diversity of genes encoding CEPs and confirmed common occurrence of *prt*H3 among *L. helveticus* strains. Analysis of 51 strains of *L. helveticus* showed that 12% of them have four genes CEP paralogs, while 8% of tested strains exhibited presence of three paralogous, and in 42% of tested bacteria two sequences encoding CEPs had been identified.

In Polish strains of *L. helveticus* analyzed, a various combinations of genes encoding CEPs (i.e. *prt*H2/*prt*H3, *prt*H/*prt*H2/*prt*H3/*prt*H4 or *prt*H3/*prt*H4) have been identified, that might indicate the different levels of the enzymes activities occurring in individual strains (Broadbent *et al.*, 2011). The study revealed also that profile III (which included the genetic variant *prt*H2 *prt*H3) was represented by the largest group (42%) of all tested *L. helveticus* strains.

The presence of prtH4 has not been confirmed in any of Polish strains. Lack of this gene sequence in *L. helveticus* strains was also reported by Miyamoto *et al.* (2015). However, some study indicated that in some *L. helveticus* strains *prt*H4 might be the only CEP gene, as in strain LHC2 derived from the USA (Jensen *et al.*, 2009).

The analysis of *L. helveticus* strains originating from Mongolia, North America and Europe confirmed a large variation with respect to cell envelope proteinase genes (Miyamoto *et al.*, 2015). This might indicate that ecological niche and environmental conditions affect

proteolytic properties of *L. helveticus* strains. Moreover, diversity of the CEPs distribution among strains might be explained by the fact that the enzymes exhibit different characteristics within different casein cleavage sites (Jensen *et al.*, 2009; Sadat-Mekmene *et al.*, 2011a). The complementary properties of different CEPs and ability of bacteria to acquire and maintain an additional CEP-encoding sequence improve adaptation to the changing environmental conditions (Genay *et al*., 2009). Furthermore, some results indicate also that differences in protease activity and amino acids metabolism of *L. helveticus* are likely to be caused by nonsense mutations that enhance the polymorphisms among the bacteria and influence the genes expression level, activity and specificity of individual enzymes involved in proteolytic reactions (Broadbent *et al.,* 2011). Therefore, the occurrence of several cell envelope proteinases in *L. helveticus* might determine the usefulness of the strains.

Conclusion

High diversity of cell-envelope associated proteinases among *L. helveticus* strains is important in formation of various compounds during proteolysis. Therefore, the results of investigations are important with regard to the possibility of forming new starters cultures for dairy industry in order to obtain the products of desired properties. The results of this study revealed significant differences in distribution of CEPs-encoding genes among *L. helveticus* strains, what seems to be a strain-dependent property. The bacterial strains demonstrated four different genetic profiles in terms of the combination variants of CEPs genes. The largest group of *L. helveticus* strains represented the combina tion of prtH2/prtH3 genes. While the sequence of *prt*H3 was the most abundant fragment of the CEP gene.

The obtained results encourage further analysis of Polish strains of *L. helveticus*. It may contribute to clarification and better understanding the relationship between genetic characteristics of CEPs-like affinity and specificity of strains to individual casein fractions.

Acknowledgements

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Literature

Broadbent J.R., H. Cai, R.L. Larsen, J.E. Hughes, D.L. Welker, V.G de Carvalho, T.A. Tompkins, Y. Ardö, F. Vogensen, A. De Lorentiis and others. 2011. Genetic diversity in proteolytic enzymes and amino acid metabolism among *Lactobacillus helveticus* strains. *J. Dairy Sci.* 94: 4313–4328.

Fortina M.G., G. Ricci, D. Mora, C. Parini and P.L. Manachini. 2001. Specific identification of *Lactobacillus helveticus* by PCR with pepC, pepN and htrA targeted primers. *FEMS Microbiol. Lett.* 198: 85–89.

Gatti M., B. Bottari, C. Lazzi, E. Neviani and G. Mucchetti. 2014. Microbial evolution in raw-milk, long-ripend cheeses produced using undefined natural whey starters. *J. Dairy Sci.* 97: 573–591.

Genay M., L. Sadat, V. Gagnaire and S. Lortal. 2009. prtH2, not prtH, is the ubiquitous cell wall proteinase gene in *Lactobacillus helveticus. FEMS Microbiol. Lett*. 75: 3238–3249.

Griffiths M.W and A.M. Tellez. 2013. *Lactobacillus helveticus*: the proteolytic system, *Front. Microbiol*. 4:1–9.

Jensen M.P., F. Vogensen and Y. Ardo. 2009. Variation in caseinolytic properties of six cheese related *Lactobacillus helveticus* strains. *Int. Dairy J.* 19: 661–668.

Kunji E.R.S., I. Mierau, A. Hagting, B. Poolman and W.N. Konings. 1996. The proteolytic systems of lactic acid bacteria. *Antonie van Leeuwenhoek* 70: 187–221.

Lozo J., I. Strahinic, M. Dalgalarrondo, J.M. Chobert, T. Haertle and L. Topisirovic. 2011. Comparative analysis of β-casein proteolysis by PrtP proteinase from *L. paracasei* subsp. *paracasei* BGHN14, PrtR proteinase from *Lactobacillus rhamnosus* BGT10 and PrtH proteinase from *Lactobacillus helveticus* BGRA43. *Int. Dairy J.* 21: 863–868.

Miyamoto M., H.M. Ueno, M. Watanabe, Y. Tatsuma, Y. Seto, T. Miyamoto and H. Nakajima. 2015. Distinctive proteolytic activity of cell envelope proteinase of *Lactobacillus helveticus* isolated from airag, a traditional Mongolian fermented mare's milk. *Int. J. Food Microbiol.* 197: 65–71.

Nejati F., M. Babaei and A. Taghi-Zadeh. 2016. Characterisation of *Lactobacillus helveticus* strains isolated from home-made dairy products in Iran, International *J. Dairy Technol.* 69: 89–95.

Nielsen M.S, T. Martinussen, B. Flambard, K.I. Sorensen and J. Otte. 2009. Peptide profiles and angiotensin-I-converting enzyme inhibitory activity of fermented milk products: effect of bacterial strain, fermentation pH, and storage time. *Int. Dairy J.* 19: 155–165.

Oberg C.J., J.R. Broadbent, M. Strickland and D.J. McMahon. 2002. Diversity in specificity of the extracellular proteinases in *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Lett. Appl. Microbiol.* 34: 455–460.

Oommen B.S., D.J. McMahon, C.J. Oberg, J.R. Broadbent and M. Strickland. 2002. Proteolytic specificity of *Lactobcillus delbrueckii* subsp. *bulgaricus* influences functional properties of mozzarella cheese. *J. Dairy Sci*. 85: 2750–2758.

Ravyts F., L. De Vuyst and F. Leroy. 2012. Bacterial diversity and functionalities in food fermentations. *Eng. Life Sci.* 12: 356–367.

Richoux R., L. Aubert, G. Roset and J.R. Kerjean. 2009. Impact of the proteolysis due to lactobacilli on the stretchability of Swiss-type cheese. *Int. J. Dairy Technol.* 89: 31–41.

Rong J., H. Zheng, M.Liu, X. Hu, T. Wang, X. Zhang, F. Jin and L. Wang. 2015. Probiotic and anti-inflammatory attributes of an isolate Lactobacillus helveticus NS8 from Mongolian fermented koumiss. *BMC Microbiology* 196: 1–11.

Sadat-Mekmene L., J. Jardin, C. Corre, D. Mollé, R. Richoux, M.M. Delage, S. Lortal and V. Gagnaire. 2011a. Simultaneous presence of PrtH and PrtH2 proteinases in *Lactobacillus helveticus* strains improves breakdown of the pure α_{s1}-Casein. *Appl. Environ. Microbiol.* 77: 179–186.

Sadat-Mekmene L., M. Genay, D. Atlan, S. Lortal and V. Gagnaire. 2011b. Original features of cell-envelope proteinases of *Lactobacillus helveticus*. A review. *Int. J. Food Microbiol.* 146: 1–13.

Sadat-Mekmene L., R. Richoux, L. Aubert-Frogerais, M.N. Madec, C. Corre, M. Piot, J. Jardin, S. le Feunteun, Lortal and V. Gag-

naire. 2013. *Lactobacillus helveticus* as a tool to change proteolysis and functionality in Swiss-type cheeses. *J. Dairy Sci.* 96: 1455–1470.
Savijoki K., H. Ingmer and P. Varmanen. 2006. Proteolytic systems of lactic acid bacteria. *Appl. Microbiol. Biotechnol.* 71: 394–406.
Savoy de Giori G. and E. M. Hébert. 2001. Methods to Determine Proteolytic Activity of Lactic Acid Bacteria, pp. 197–202. In: Spencer J.F.T. and A.L. Ragout de Spencer (eds.). Methods in Biotechnology, *Food Microbiology Protocols*. Humana Press Inc. Totowa, New Jersey.
Smeianov V.V., V.P. Wechter, J.R. Broadbent, J.E. Hughes, B.T. Rodriguez, T.K. Christensen, Y. Ardo and J.L. Steele. 2007. Comparative high-density microarray analysis of gene expression during growth of *Lactobacillus helveticus* in milk versus rich culture medium. *Appl. Environ. Microbiol*. 73: 2661–2672.
Soeryapranata E., J.R. Powers and G. Ünlü. 2007. Cloning and Characterization of Debittering Peptidases, PepE, PepO, PepO2, PepO3 and PepN, of *Lactobacillus helveticus* WSU 19. *Int. Dairy J*. 17: 1096–1106.
Wakai, T. and N. Yamamoto. 2012. Antihypertensive peptides specific to *Lactobacillus helveticus* fermented milk. pp. 159–172. In: Sammour R. H. (ed.) *Biotechnology – Molecular Studies and Novel Applications for Improved Quality of Human Life*. In Tech Europe, Rijeka, Croatia.
Waśko, D. Szwajgier and M. Polak-Berecka. 2014. The role of ferulic acid esterase in the growth of *Lactobacillus helveticus* in the presence of phenolic acids and their derivatives. *Euro. Food Res. Technol.* 238: 299–236.
Widyastuti Y., Rohmatussolihat and A. Febrisiantosa. 2014. The role of lactic acid bacteria in milk fermentation. *FNS* 5: 435–442.

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ORIGINAL PAPER

The Usefulness of Chromogenic Media for Qualitative and Semi-Quantitative Diagnostic of Urinary Tract Infections

ELŻBIETA M. STEFANIUK*

Department of Epidemiology and Clinical Microbiology, National Medicines Institute, Warsaw, Poland

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Abstract

The aim of this study was to evaluate the usefulness of chromogenic media for isolation of bacteria from urine and direct identification of UTI pathogens. A total of 100 urine specimens were inoculated on blood agar and MacConkey agar as a reference method and on the following media to be tested: chromID® CPS® Elite (CPSE, bioMérieux), CHROMagar™ Orientation (BioMaxima), BD CHROMagar Orientation Medium (ORI, Becton Dickinson), CHROMagar™ Orientation (ORIE, Graso) and Brillance UTI Clarity Agar (UTI C, Oxoid). After a 24-hour incubation period, 47 Gram-positive cocci and 62 Gram-negative rods were observed. The specificity and sensitivity of all chromogenic media was 97.3% and 93.5% respectively for qualitative diagnostic; and 81.9% and 81.3% respectively for semi-quantitative diagnostic. The mean PPV and NPV of the chromogenic media were 98.7% and 87.7% for qualitative UTI diagnostic, and 90.9% and 71.9% respectively for semi-quantitative diagnostic.

Key words: chromogenic media, qualitative and semi-quantitative microbiological diagnostic, urinary tract infections (UTI)

Urinary tract infections (UTIs) belong to the most common infections both in community and hospital settings. They account for 10–20% of all infections treated in primary care and 30–40% of infections treated in hospitals. *Escherichia coli* remains the predominant uropathogen (80%) isolated in the acute community-acquired uncomplicated infections. Uncomplicated cystitis and pyelonephritis may also be caused by other species of Enterobacteriaceae rods (*Klebsiella* spp., *Proteus* spp., *Enterobacter* spp.) or, less frequently, by coagulase-negative staphyloccocci and enterococci. The etiology of UTI also depends on the patient's age, gender and many other factors predisposing them to urinary tract infection as: urinary/fecal incontinence, nephritis, kidney stones, prostate hypertrophy, renal insufficiency, diabetes, kidney empyema, polycystic kidneys and kidney cancer (Pezzlo, 2014; Flores-Mireles *et al.*, 2015; Stefaniuk *et al.*, 2016b).

Urine samples constitute a large share in the daily workload of microbiological laboratories. A combination of blood agars, such as Columbia agar and MacConkey agar, are traditionally widely used for urine culture (Green, 2009; Akter *et al.*, 2014). These are non-selective media capable of supporting the growth of most pathogens. MacConkey agar is able to identify aerobic Gram-negative bacteria by detecting lactose utilisation, but is not capable for detecting the mixed Gram-negative cultures. The pathogens grow on chromogenic media for UTI diagnostics by forming colonies of a characteristic colour. The pathogen identification is based on chromogenic substrates contained in the media to detect the bacterial enzymes, mainly β-galactosidase, β-glucosidase, β-glucuronidase (the bacteria grow in the form of pink-red or blue-green colonies, respectively) and tryptophan deaminase, which is characteristic for the *Proteae* group of bacteria (*Proteus-Morganella-Providencia*). These pathogens appear on chromogenic media as brownish colonies (Fallon *et al.*, 2003; Pezzlo, 2014; Yarbrough *et al.*, 2016).

The aim of the study was to evaluate the usefulness of chromogenic media in detecting bacteria from urine and identifying UTI pathogens directly. The media were evaluated by using 100 urine specimens sent for routine diagnostic from paediatric and adult patients of two Polish hospitals: the Poviate Hospital in Wołomin, and the Baby Jesus University Hospital in Warsaw, between May and September 2016. Five chromogenic culture media were tested, namely chromID® CPS® Elite (CPSE; bioMérieux, France), BD CHROMagar Orientation Medium (ORI; Becton Dickinson, Germany), Brillance UTI Clarity Agar (UTI C; Oxoid, USA), CHROMagar™ Orientation (BMagar; BioMaxima,

* Corresponding author: E.M. Stefaniuk, Department of Epidemiology and Clinical Microbiology, National Medicines Institute, Warsaw, Poland; **e-mail: elzbieta.stefaniuk@gmail.com**

Poland), CHROMagar™ Orientation (ORIE; Graso, Poland). Blood agar (Columbia agar enriched with 5% sheep blood (BAP; Becton Dickinson, Germany) and MacConkey agar (MAC; Becton Dickinson, Germany) served as the reference media. All media were inoculated with a 0.01 ml disposable plastic loop as prescribed (Sharp *et al*., 2009). The plates were incubated and the bacterial colony characteristics were assessed after 18 to 24 hours at the Department of Epidemiology and Clinical Microbiology (DECM) of the National Medicines Institute in Warsaw, Poland. Additionally, each batch was tested for typical colony appearance and growth with reference strains of *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *K. pneumoniae* ATCC 13883, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *P. mirabilis* ATCC 29245.

Presumptive identification of isolates using the manufacturer's colour criteria was compared with the biochemical identification of bacteria Vitek 2 Compact system (bioMérieux, Marcy l'Etoile France) as a reference method, but complementary tests such as microscopic examination by Gram stain method, indol, oxidase and catalase detection (Perry, 2017) were also performed. Bacterial growth was recorded semi-quantitatively based on the colony count. All plates were recorded as having no growth (NG), 1, 2, 3, 4 or 5 colony types according to their morphology and colour. The number of each colony type was also recorded to facilitate the identification of pathogens in mixed cultures; 10^2 CFU/ml signified a growth of < 10 CFU on a plate; 10^3 CFU/ml signified 10–99 CFU on a plate, *etc*. (Sharp *et al*, 2009). The qualitative and quantitative urine culture results were compared with the results of urine culture on BAP and MAC. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the chromogenic media were calculated according to the guidelines of the American Society of Microbiology (ASM) (Sharp and Clark, 2009). For the analysis of the results, all data was entered into an Excel spreadsheet.

In forty-one out of 100 (41%) urine samples no microbial growth (NG) was observed on any of the media used in the study. In the remaining 59 urine samples, 1 to 5 distinct microorganisms were cultured. Sixty-two Gram-negative rods (Enterobacteriaceae n = 61 and non-fermenting rods n = 1) and 47 Gram-positive cocci (enterococci n = 22; staphylococci n = 21; streptococci n = 4) were isolated from the urine samples. All of the isolates were screened for their colony colours using the colour criteria provided by manufacturers. Table I shows the analysis of bacterial growth on chromogenic media as compared with the reference method. *E. coli* constituted 53.2% (n = 33) of the Gram-negative rods collected from urine samples. Differences in *E. coli* growth were observed in three cases (no. 80, 3866 and 4181). *Enterobacter cloacae* was characterized by KES colonies (*Klebsiella, Enterobacter, Serratia*), while colonies of *Citrobacter freundii* resembled the *E. coli* ones in colour. None of the tested chromogenic media allowed identification of *Acinetobacter* spp. colonies by colour; the colony colour suggested the *Proteae* group.

As regards the Gram-positive cocci group, four streptococcal isolates grew on all media used in this study, but various results were obtained for twelve isolates – four *Enterococcus faecalis* (samples no.: 91, 4181, 12935, 3866) and eight staphylococcal isolates *(S. epidermidis* n = 2; *S. haemolyticus* n = 2; *S. hominis* n = 2 and *S. warnerii* n = 1). Colonies of *Enterococcus* spp. are similar in colour, their identification is therefore only possible to the level of the genus. Colonies of *Streptococcus* spp. appear on chromogenic media as blue. Streptococci and enterococci are easily distinguishable from each other, since the *Enterococcus* spp. colonies are blue-green and grow larger than streptococci colonies.

Monoculture was recorded in 31 (52.5%) "positive" samples; two morphologically different bacterial isolates were cultured in 12 (20.3%) urine samples; three different strains were found in 7 (11.9%) samples, four – in 7 (11.9%) samples, whereas one cultured sample (1.7%) yielded five different microorganisms. An analysis of the number of samples containing mixed bacterial population showed that all five chromogenic media evaluated in this study allowed for pre-differentiation of pathogen types when compared to the reference method. A direct comparison of these chromogenic media showed minor discrepancies between them in detecting all microorganisms when mixed culture was observed (Table II).

Table III summarises the sensitivity, specificity, PPV and NPV of chromogenic media for UTI diagnostic with qualitative and semi-quantitative colony count (for all pathogens) and the sensitivity for *E. coli* only. The specificity and sensitivity of chromogenic media calculated for the entire study group was 97.3% and 93.5% respectively for the qualitative diagnostic, whereas for the semi-quantitative diagnostic the values mentioned above were 81.9% and 81.3% respectively. The mean value of PPV and NPV of the chromogenic media for the qualitative UTI diagnosis was 98.7% and 87.7 % respectively, and for semi-quantitative diagnosis, 90.9% and 71.9% respectively. The difference between the chromogenic media in qualitative and semi-quantitative colony count methods was not statistically significant ($p > 0.99$ and $p = 0.9$ respectively). The sensitivity main values (%) of chromogenic media for qualitative and semi-quantitative cultures for *E. coli* were 96.0% and 90.6% respectively. In qualitative UTI diagnostic, the ORIE achieved the highest sensitivity in the study, amounting to 97.2%. The CPSE and ORIE media exhibited the highest sensitivity for the semi-quantitative

Table I
Comparison of microbial growth on chromogenic media *vs*. reference method.

Bacterial strains			Number of isolates (n)						
			The reference method		Chromogenic media				
Family or genus (n)	Species (by VITEK 2 Compact) and isolates number		Columbia Agar + 5% sheep blood (COL)	MacConkey Agar (MAC)	ChromID® CPS® Elite (CPSE, bioMérieux)	BD CHROMagar Orientation Medium (ORI, Becton Dickinson)	Brillance UTI Clarity Agar (UTI C, Oxoid)	CHROMagar™ Orientation (BioMaxima)	CHROMagar™ Orientation (ORIE, Graso)
Gram-negative rods (n = 62)									
Enterobacteriaceae (n = 61)	*Citrobacter freundii*	1	1	1	1	1	1	1	1
	Enterobacter cloacae	1	1	1	1	1	1	1	1
	Escherichia coli	33	32	31	32	30	33	31	33
	Klebsiella pneumoniae	16	16	15	16	16	16	16	16
	Klebsiella oxytoca	1	1	1	1	1	1	1	1
	Morganella morganii	2	2	2	2	2	2	2	2
	Proteus mirabilis	6	6	6	6	6	6	6	6
	Proteus vulgaris	1	1	1	1	1	1	1	1
Non-fermenting rods (n = 1)	*Acinetobacter lwofii*	1	1	1	0	1	1	1	1
Gram-positive cocci (n = 47)									
Enterococci (n = 22)	*Enterococcus avium*	1	1	0	1	1	1	1	1
	Enterococcus faecalis	20	20	0	18	20	18	19	20
	Enterococcus hirae	1	1	0	1	1	1	1	1
Staphylococci (n = 21)	*Staphylococcus epidermidis*	9	9	0	8	6	8	8	9
	Staphylococcus haemolyticus	4	4	0	4	3	3	3	3
	Staphylococcus hominis	7	7	0	6	6	6	6	6
	Staphylococcus warnerii	1	1	0	1	0	1	1	0
Streptococci (n = 4)	*Streptococcus agalactiae*	1	1	0	1	1	1	1	1
	Streptococcus viridans group	3	3	0	3	3	3	3	3

Table II
Detection of bacteria in mono- and mixed urine culture on chromogenic media for UTI diagnosis.

Number of isolates in a clinical specimen	Number of clinical specimens (urine)	Number of all isolates in all clinical specimens	Total number of isolates (% of the number cultured on reference media)				
			Chromogenic media				
			ChromID CPS Elite (CPSE, bioMérieux)	BD CHROMagar Orientation Medium (ORI, Becton Dickinson)	Brillance UTI Clarity Agar (UTI C, Oxoid)	CHROMagarTM Orientation (BioMaxima)	CHROMagarTM Orientation (ORIE, Graso)
1	31	31	30 (96.8%)†	29 (93.6%)†	31 (100%)	30 (96.6%)†	31 (100%)
2	12	24	23 (95.8%)†	22 (91.7%)†, ¶	22 (91.7%)§	23 (95.8%)¶	24 (100%)
3	7	21	21 (100%)	18 (85.7%)†, ‡, ††	17 (81.0%)†, ‡, §	19 (90.5%)†	20 (95.3%)†
4	7	28	25 (89.3%)†, ¶	25 (89.3%)†, ‡, ¶	27 (96.4%)†	25 (89.3%)†, §, ¶	26 (92.6%)†, ‡
5	1	5	5 (100%)	5 (100%)	5 (100%)	5 (100%)	5 (100%)

† – no growth of *Staphylococcus* spp. cultured on sheep blood-enriched Columbia agar with a colony count of 10^2 CFU/ml (clinical specimens no. 78, 79, 89, 3855, 3873, 12929, 12935)
‡ – no growth of *Staphylococcus* spp. cultured on sheep blood-enriched Columbia agar with a colony count of 10^3 CFU/ml (clinical specimen no. 4181)
§ – no growth of *Enterococccus* spp. cultured on sheep blood-enriched Columbia agar with a colony count of 10^2 CFU/ml (clinical specimens no. 12935, 4181, 3866)
¶ – no growth of Gram-negative rods cultured on sheep blood-enriched Columbia agar or MacConkey agar with a colony count of 10^2 CFU/ml (clinical specimens no. 3866, 80)
†† – no growth of Gram-negative rods cultured on sheep blood-enriched Columbia agar and MacConkey agar with a colony count of 10^3 CFU/ml (clinical specimen no. 4181)

Table III
Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) of chromogenic media for UTI diagnosis.

Parameters	Chromogenic media					
	ChromID® CPS® Elite (CPSE, bioMérieux)	BD CHROMagar Orientation Medium (ORI, Becton Dickinson)	Brillance UTI Clarity Agar (UTI C, Oxoid)	CHROMagar™ Orientation (BioMaxima)	CHROMagar™ Orientation (ORIE, Graso)	Mean value
Qualitative cultures for all detected pathogens ($p > 0.99$)						
Sensitivity (%)	94.7	91.7	89.6	94.4	97.2	93.5
Specificity (%)	97.7	95.5	97.7	97.7	97.7	97.3
Positive predictive value (PPV) (%)	98.9	98.0	98.4	99.0	99.1	98.7
Negative predictive value (NPV) (%)	89.4	82.4	85.7	87.8	93.3	87.7
Qualitative cultures of *E. coli*						
Sensitivity (%)	96.8	90.1	100	93.3	100	96.0
Semi-quantitative cultures for all detected pathogens ($p = 0.9$)						
Sensitivity (%)	85.2	80.2	75.0	81.0	85.1	81.3
Specificity (%)	85.7	82.4	91.3	82.7	82.4	84.9
Positive predictive value (PPV) (%)	91.5	90.0	92.3	90.0	90.5	90.9
Negative predictive value (NPV) (%)	76.4	67.7	72.4	69.4	73.7	71.9
Semi-quantitative cultures of *E. coli*						
Sensitivity (%)	93.1	84.0	95.2	87.1	93.6	90.6

urine culture (85.2% and 85.1% respectively). The UTI C and CPSE media had the highest PPV for the semi-quantitative method, achieving 92.3% and 91.5% respectively, whereas BMagar had the lowest PPV of all (90.0%). The CPSE and ORIE media had the highest NPV for the semi-quantitative method, achieving 76.4% and 73.7% respectively, whereas the lowest NPV of all (70.0%) was recorded for BMagar and ORI.

A detailed analysis of the semi-quantitative assay of different bacteria species on chromogenic media when compared to the BAC and MAC reference media demonstrated a tendency for decreased colony count on some media, yet the differences were not statistically significant. The count of *Stapylococcus* spp. was lower on all chromogenic media. Enteroccocal growth was inhibited in three of the five tested media (UTI C, BMagar and

ORI). A diminished colony count (CFU/ml) of Enterobacteriaceae was observed on ORI and UTI C media.

Microbiology laboratories always attempt to reduce the turnaround time and the cost of pathogens identification and their susceptibility testing (Strauss and Bourbeau, 2015). In recent years, chromogenic culture media have been widely used in clinical microbial diagnostic for detection and preliminary identification of pathogens and antimicrobial resistance mechanisms (Fillius *et al.*, 2003; Kuch *et al.*, 2009; Stefaniuk *et al.*, 2016a; Yarbrough *et al.*, 2016). In our study, apart from the results obtained using the three chromogenic media used all over the world (CPSE, ORI and UTI C), we present results obtained using two media manufactured in Poland: BMagar – BioMaxima and ORIE – Graso, which are offered for a lower price. Moreover, along with the qualitative analysis of the cultures obtained on these media, a thorough qualitative analysis of the specimens of the urine samples was conducted. Many studies have shown identical or even better results regarding the detection and identification of UTI pathogens using various chromogenic media over conventional media (Aspevall *et al.*, 2002; Scarparo *et al.*, 2002). The interpretation of mixed cultures of urines is difficult. To diagnose the mixed urine cultures properly, a laboratory needs additional information about the patient. As earlier studies have suggested (Price *et al.*, 2016), chromogenic media are more effective in preventing the swarming of *Proteus* species when compared to conventional media: blood agar or MacConkey agar. For this reason chromogenic media have enjoyed great popularity. Our research adds to this knowledge by demonstrating the utility of chromogenic media in mixed culture detection. It was shown, however, that depending on the media type, colonies of the same isolate might take different colours. Thus, in the vast majority of cases a colony colour only indicates a microbial group rather than a species. This claim is supported by our findings related to the Enterobacteriaceae, in particular *E. coli*, *E. cloacae* and *C. freundii*, as well as *A. lwofii* isolates. Similarly, the results of our semi-quantitative assay along with the diminished colony count on chromogenic media, point to the need to use chromogenic media as "auxiliary" to the conventional method based on blood agar and MacConkey agar.

ASM recommends culturing urinary samples for colony count on blood agar media only. Other media can be used for semi-quantitative tests, after being evaluated for possible inhibition of bacteria growth. MAC and chromogenic media can limit the growth of bacteria but according to the reference values, the recovery usually amounts to no less than 30%, (Pasek, 1999; Sharp and Clark, 2009; Sharp *et al.*, 2009). Such results were observed for *E. coli* no. 3866 and 4181 and *S. epidermidis* no. 4181. In other cases (*E. coli* no. 80; *E. faecalis* – no. 4181, 12935 and 3866) the lack of growth was associated with the limitations of the method. The 10 µl loop allows the detection of colony counts between 100 and 1000 CFU/ml. In urine containing 10^2 CFU/ml, one loopful transferred onto an agar medium may only contain one colony per plate. When the bacteria are unevenly distributed in the sample, or the density of bacteria is low, bacterial growth on agar plates may not be detected (Chan, 2016). However, many authors concluded that chromogenic media might potentially be used as the single medium for the culture of urine samples (Chang *et al.*, 2008; Jolkkonen *et al.*, 2010). Ojanen T. *et al.* (2016) analysed the results of quantitative cultures of urine samples from laboratories participating in the External Quality Assessment (EQA) in Finland to evaluate the reliability of quantitative urine culture. Among laboratories routinely using chromogenic media, up to 87% obtained the correct results. Interlaboratory comparative studies have shown that the growth of *S. agalactiae* on chromogenic media is significantly better compared to the conventional, non-chromogenic media.

Due to the high number of urine culture specimens processed annually, laboratories are constantly seeking ways to improve their effectiveness. Automated instruments for dispensing the urine samples onto culture plates have been introduced. Due to the high prices of new technical solutions, they are only utilised in big laboratories that diagnose a large number of specimens. In small laboratories, the basic research methods and techniques in UTI diagnostics, such as urine culture and counting colonies are still used. Using chromogenic media appears to be a good alternative to conventional microbial diagnostic of UTI, yet, the user has to know the limitations of chromogenic media. The benefits of using chromogenic media in UTI diagnostics will most likely be seen in a non-hospital laboratory, because the most frequently isolated species from uncomplicated UTI infections is *E. coli* (Pezzlo, 2014; Rigaill *et al.*, 2015). Stefaniuk *et al.* (2016b) pointed out that in Poland *E. coli* was responsible for 80.6% of cases of uncomplicated infections and 65.8% of the complicated ones.

In conclusion, all the chromogenic media tested in our study are attractive and easy-to-use screening media that considerably reduce the daily workload and the number of identification kits required.

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Literature

Akter L., R. Haque and M.A. Salam. 2014. Comparative evaluation of chromogenic agar medium and conventional culture system for isolation and presumptive identification of uropathogens. *Pak. J. Med. Sci.* 5: 1033–1038.

Aspevall O., B. Osterman, R. Dittmer, L. Stén, E. Lindbäck and U. Forsum. 2002. Performance of four chromogenic urine culture media after one or two days of incubation compared with reference media. *J. Clin. Microbiol.* 40: 1500–1503.

Chan W.W. 2016. Urine cultures, pp. 3.12–1–3.12–2. In: Leber A.L. (ed.). *Clinical microbiology procedures handbook*. ASM Press, Washington, DC.

Chang J.C., M.L. Chien, H.M. Chen, J.J. Yan and J.J. Wu. 2008. Comparison of CPS ID 3 and CHROMagar Orientation chromogenic agars with standard biplate technique for culture of clinical urine samples. *J. Microbiol. Immunol. Infect.* 41: 422–427.

Fallon D., G. Ackland, N. Andrews, D. Frodsham, S. Howe, K. Howells, K.J. Nye and R.E. Warren. 2003. A comparison of the performance of commercially available chromogenic agars for the isolation and presumptive identification of organisms from urine. *J. Clin. Pathol.* 56: 608–612.

Filius P.M.G., D. van Netten, P.J.E. Roovers, A.G. Vulto, I.C. Gyssens, H.A. Verbrugh and H.P. Endtz. 2003. Comparative evaluation of three chromogenic agars for detection and rapid identification of aerobic Gram-negative bacteria in the normal intestinal microflora. *Clin. Microbiol. Infect.* 9: 912–918.

Flores-Mireles A.L., J.N. Walker, M. Caparon and S.J. Hultgren. 2015. Urinary tract infections: epidemiology, mechanisms of infection, and treatment options. *Nat. Rev. Microbiol.* 13: 269–284.

Green L.H. 2009. Culturing and Preserving Microorganisms, pp. 31–35. In: Goldman E. and L.H. Green (eds). *Practical Handbook of Microbiology.* CRC Press, Boca Raton, FL.

Jolkkonen S., E.L. Paattiniemi, P. Kärpänoja and H. Sarkkinen. 2010. Screening of urine samples by flow cytometry reduces the need for culture. *J. Clin. Microbiol.* 48: 3117–3121.

Kuch A., E. Stefaniuk, T. Ozorowski and W. Hryniewicz. 2009. New selective and differential chromogenic agar medium, chromID VRE, for screening vancomycin-resistant *Enterococcus* species. *J. Microbiol. Methods.* 77: 124–126.

Ojanen T., S. Kiiskinen, Y. Björkman, H. Laitinen, M.J. Virtanen and A. Siitonen. 2016. Use of external quality assessment to evaluate the reliability of quantitative urine culture. *Arch. Clin. Microbiol.* 7: 2–7.

Pasek W. 1999. Validation of microbiological methods (in Polish). *Pharmaceutica.* 8: 4–6.

Perry J.D. 2017. A decade of development of chromogenic culture media for clinical microbiology in an era of molecular diagnostics. *Clin. Microbiol. Rev.* 30: 449–479.

Pezzlo M.A. 2014. Laboratory diagnosis of urinary tract infections: guidelines, challenges, and innovations. *Clin. Microbiol. Newsletter*. 36: 87–93.

Price T.K., T. Dune, E.E. Hilt, K.J. Thomas-White, S. Kliethermes, C. Brincat, L. Brubaker, A.J. Wolfe, E.R. Mueller and P.C. Schreckenberger. 2016. The clinical urine culture: enhanced techniques improve detection of clinically relevant microorganisms. *J. Clin. Microbiol.* 54: 1216–1222.

Rigaill J., P.O. Verhoeven, C. Mahinc, M. Jeraiby, F. Grattard, N. Fonsale, B. Pozzetto and A. Carricajo. 2015. Evaluation of new bioMérieux chromogenic CPS media for detection of urinary tract pathogens. *J. Clin. Microbiol.* 53: 2701–2702.

Scarparo C., P. Piccolo, P. Ricordi and M. Scagnelli. 2002. Comparative evaluation of two commercial chromogenic media for detection and presumptive identification of urinary tract pathogens. *Eur. J. Clin. Microbiol. Infect. Dis.* 21: 283–289.

Sharp S.E., Y.S. McCarter, E.M. Burd, G.S. Hall and M. Zervos (eds). 2009. *Cumitech 2C, Laboratory Diagnosis of Urinary Tract Infections.* ASM Press, Washington, DC.

Sharp S.E. and R.B. Clark (eds). 2009. *Cumitech 31A, Verification and validation of procedures in the Clinical Microbiology Laboratory*. ASM Press. Washington, DC.

Stefaniuk E., A. Baraniak, M. Fortuna, W. Hryniewicz. 2016. Usefulness of CHROMagar Candida medium, biochemical methods – API ID32C and VITEK 2 Compact and two MALDI-TOF MS Systems for *Candida* spp. identification. *Polish J. Microbiol.* 65: 111–114.

Stefaniuk E., K. Bosacka and W. Hryniewicz. 2016. Etiology and antibiotic susceptibility of bacterial pathogens responsible for community-acquired urinary tract infections in Poland. *Eur. J. Clin. Microbiol. Infect. Dis.* 35: 1363–1369.

Strauss S. and P.P. Bourbeau. 2015. Impact of introduction of the BD Kiestra InoquIA on urine culture results in a hospital clinical microbiology laboratory. *J. Clin. Microbiol.* 53: 1736–1740.

Yarbrough M.L., M.A. Wallace, C. Marshall, E. Mathias and A.-A.D. Burnham. 2016. Culture of urine specimens by use of chromID CPS Elite medium can expedite *Escherichia coli* identification and reduce hands-on time in the clinical laboratory. *J. Clin. Microbiol.* 54: 2767–2773.

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SHORT COMMUNICATION

Identification of Pathogenicity of *Yersinia enterocolitica* in Pig Tonsils Using the Real-Time PCR

MILENA A. STACHELSKA

Lomza State University of Applied Sciences, Institute of Food Technology and Gastronomy, Lomza, Poland

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Abstract

The application of DNA-based methods enables to identify *Yersinia enterocolitica* carrying the *ail*-gene with a greater sensitivity compared to culture methods and biochemical tests used for detection of pathogenic *Y. enterocolitica* in animal and food samples. In this study, 100 samples of pig tonsils were examined, among which 17 were positive for the *ail* gene. Additionally, biochemical tests and RT-PCR showed that nine *Y. enterocolitica* isolates carried the *ail*-gene. Two *Y. enterocolitica* isolates of 1A biotype had the *ail* gene. The results demonstrated the usefulness of RT-PCR method applied for detection of potentially pathogenic, possessing the *ail* gene *Y. enterocolitica* in the material examined.

Key words: RT-PCR, *Yersinia enterocolitica*, pathogen specific gene (*ail*), biochemical testing, biotyping

Yersinia enterocolitica is a rod-shaped bacteria belonging to the family Enterobacteriaceae. *Yersinia* spp. contains 17 species. Three of them are pathogenic to humans, as follows: *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (Thoerner *et al.*, 2003; Bolton *et al.*, 2013). The identification of *Y. enterocolitica* is based on the amplification of pathogen specific gene (*ail*) in the presence of the Internal Control (IC) with specific primers and probes labelled with fluorescent dyes. The main animal reservoir of *Y. enterocolitica* strains constitutes pig tonsils (Tennant *et al.*, 2003; Wang *et al.*, 2009). The bacteria attack the lymph tissues during infection due to a 70 kb virulence plasmid (pYV) that encode proteins that participate in infection of these tissues. *Y. enterocolitica* is an enteric pathogen of six biotypes: 1A, 1B, 2, 3, 4, and 5. Different bioserotypes are regarded to be pathogenic. The isolates of biotype 1A do not possess the virulence-associated pYV-encoded genes and most of chromosomal virulence markers, including the *ail* (attachment and invasion locus) gene.

Over the last years, there have been many molecular methods designed to improve and accelerate the detection procedures. The comparison of conventional and molecular detection methods of *Y. enterocolitica* showed a significantly higher sensitivity of the molecular methods. The most significant steps are: i) an efficient enrichment procedure for pathogenic *Y. enterocolitica* isolates in the appropriate medium and ii) a DNA preparation protocol to remove any inhibitors of the subsequent molecular reactions (Bonardi *et al.,* 2014). Enrichment procedures enable the discrimination between viable and non-viable bacterial cells, thereby contributing to the diminished risk of false positive results (Lambertz *et al.,* 2007). The aim of this study was to show the usefulness of RT-PCR assay (PowerChek™ *Yersinia enterocolitica* RT-PCR Detection System (KogeneBiotech, Germany)) for fast detection of potentially pathogenic, possessing the *ail* gene *Y. enterocolitica* in pig tonsils.

Pig tonsils from 100 pigs, which were slaughtered in one abattoir in podlaskie voivodeship in Poland, were taken during 8 sampling visits. Pig tonsils came from pigs, which were slaughtered at the age of 150–170 days. Pigs were from 18 herds. Tonsil samples were aseptically cut into small pieces, and 10 g of tonsils were put into sterile stomacher bag. Samples were homogenized with 90 ml of 0.1% peptone water (Oxoid, UK) for 2 min in a stomacher (Colworth Stomacher 400, Seward Ltd, London, UK). 0.1 ml of such homogenate was plated on CIN agar plates by a spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain). CIN agar plates are cefsulodin-irgasan-novobiocin agar plates (Yersinia Selective Agar Base and Yersinia Selective Supplement, Oxoid, UK). CIN agar plates were incubated at 30°C for 24 h and investigated for characteristic *Yersinia* colonies using a stereomicroscope with Henry illumination (Olympus).

* Corresponding author: M.A. Stachelska, Lomza State University of Applied Sciences, Institute of Food Technology and Gastronomy, Lomza, Poland; **e-mail: mstachelska@pwsip.edu.pl**

To achieve the accurate assessment of the pathogenicity of *Y. enterocolitica* belonging to biogroup 1A with *ail* gene, the invasion and adhesion assay onto Hep-2 cells (human epidermis carcinoma cells, larynx) with 2006RAT and SDWL-003 were carried out. Two isolates of *Y. enterocolitica* with various virulence potentials were chosen as the reference isolates: weakly virulent (bioserotype 3/O:3, isolate NX-SA98-837) and highly virulent (bioserotype 1B/O:8, isolate Ye92010) (Table II). Invasion and adhesion assays were performed as described previously by Wang *et al.* (2008). Experiments were repeated three times. The assay for each isolate was repeated two times in one experiment. The results were analyzed by the T test; a critical P value of 0.05 was used as statistically significant.

The cultures grown in tryptic soy broth were taken for purification of DNA for RT-PCR analysis. The total reaction volume was 20 µl, the volume of DNA was 5 µl, Primer/Probe Mix was 4 µl, 2X RT-PCR Master Mix was 10 µl, PCR water (negative control) was 1 µl. The fluorescent curves were analysed on HEX fluorescence detection channels (Table I). Analysing RT-PCR results assessed the presence or absence of *Y. enterocolitica* specific gene.

The biochemical tests were carried out to check the pathogenicity of *Yersinia* isolates. Nine out of 100 samples of tonsils gave positive results for pathogenic *Y. enterocolitica.* The growth on Bile Esculine Agar enabled to distinguish the pathogenic from non-pathogenic *Y. enterocolitica* isolates. A black halo around the colonies indicates a positive reaction. Any pathogenic *Y. enterocolitica* were found. Another test was carried out on Kligler Iron Agar. A butt was yellow and a slant was red what showed that the growth of *Y. enterocolitica* was observed. *Y. enterocolitica* is glucose positive and lactose negative, does not form H_2S or gas. The pathogenicity of Yersinia isolates was also tested on Urea Agar. Pink-violet or red-pink color of the slant indicated a positive urease reaction. An orange-yellow color indicated a negative urease reaction. As the slant was red-pink, the growth of *Y. enterocolitica* was observed, which indicated a positive urease reaction.

Table I
Detection of pathogenic *Yersinia enterocolitica* in pig tonsils with RT-PCR.

Number of samples	Source	Ct ranges in HEX channel for positive results	Ct ranges in HEX channel for negative results
Positive control	Control DNA	28.89	–
17	Pig tonsils	25.67–36.17	–
83	Pig tonsils	–	37.23–37.63
Negative control	PCR water	–	37.34

We used RT-PCR test to confirm the presence of potentially pathogenic *Y. enterocolitica* isolates possessing the *ail* gene in the material examined. The probe-based RT-PCR assay for detection of *ail*-positive *Y. enterocolitica* was found to be specific for all potentially pathogenic isolates present in pig tonsil samples (Table I). 17 out of 100 samples of tonsils gave positive results for pathogenic *Y. enterocolitica* as indicated a positive reaction with Ct values between 25.67 and 36.17. Remaining negative samples showed Ct values between 37.23–37.63. Relatively low Ct values (≤ 36.17) in the positive samples of pig tonsils for *ail* gene meant that tonsils were highly contaminated (Table I).

All the *ail*-positive isolates of *Y. enterocolitica* obtained using the culture method were subjected to biotyping. The API 20E encode isolates 2006RAT and SDWL-003 with numbers 1.155.723 and 1.114.721, respectively. In accordance to the code display results, they were all *Y. enterocolitica*. The isolates 2006RAT and SDWL-003 had typical biochemical profile for biotype 1A according to the biotyping schema. They were Tween-esterase positive, esculin positive, pyrazinamidase positive, indol positive, xylose positive and trehalose positive. The *ail*-positive isolate of *Y. enterocolitica* Ye92010 had typical biochemical profile for biotype 1B as follows: tween-esterase positive, esculin negative, pirazinamidase negative, indol positive, xylose positive and trehalose positive. The *ail*-positive isolate of *Y. enterocolitica* NX-SA98-837 had typical biochemical profile for biotype 3. It was Tween-esterase negative, esculin negative, pirazinamidase negative, indol negative, xylose positive and trehalose positive. We assessed biotypes of the isolates of *Y. enterocolitica* examined according to Wauters *et al.* scheme (1987) (Table II).

The adhesion and invasion properties of *Y. enterocolitica* biotype 1A containing the *ail* gene in comparison to biotype 3 were evaluated. The results of this test were presented in Table III. The results of the cell adhesion and invasion assay indicated the differences between 2006RAT, SDWL-003, a weakly virulent isolate NX-SA98-837 and a highly virulent isolate Ye9201. The isolates were arranged according to their ability of adhesion to Hep-2 cells. The highest number of NX-SA98-837 cells was able to adhere to Hep-2 cells and the lowest number of SDWL-003 cells was able to adhere to Hep-2 cells (Table III). The significant differences ($P < 0.05$) were observed for NX-SA98-837 and Ye92010 in comparison to SDWL-003. The gentamicin treatment in the invasion test revealed that number of live 2006RAT cells was significantly higher in comparison to Ye92010 cells. Moreover, no significant difference was observed for the survival of SDWL-003 cells. It means that 2006RAT was more invasive than SDWL-003 and highly virulent 1B/O:8 isolate Ye92010 (Table III).

Table II
Assessment of biotypes of *Yersinia enterocolitica* isolates according to Wauters *et al.* scheme (1987).

Strains	Biochemical tests									Biotype
	Lipase (Tween-esterase)	Esculin/ salicin 24 h	Indole	Xylose	Trehalose/ NO_3	Pyrazinamidase	β-D-Glucosidase	Voges-Proskauer	Proline peptidase	
2006RAT	+	+	+	+	+	+	+	+	V[1]	1A
SDWL-003	+	+	+	+	+	+	+	+	V[1]	1A
Ye92010	+	–	+	+	+	–	–	+	–	1B
NX-SA98-837	–	–	–	+	+	–	–	+	–	3

V[1] – variable

Table III
The adhesion and invasion properties of *Yersinia enterocolitica* biotype 1A containing the *ail* gene in comparison to biotype 3.

Isolate	Biotype	A number of bacterial cells adhering to Hep-2 cells	A number of bacterial cells surviving within Hep-2 cells
2006RAT	1A	$5\,000 \pm 10^{T}$	$10\,000 \pm 12$
SDWL-003	1A	$1\,000 \pm 10$	500 ± 7
Ye92010	1B	$750\,000 \pm 23$	$4\,500 \pm 10$
NX-SA98-837	3	$2\,100\,000 \pm 36$	$32\,000 \pm 16$

[T] Standard deviation from three separate experiments $P < 0.05$

In our work we examined prevalence of pathogenic *Y. enterocolitica* isolates in pig tonsils using RT-PCR and biochemical tests. The higher percentage of pathogenic isolates was detected using RT-PCR. In RT-PCR, the isolates possessing the *ail* gene were classified as pathogenic, and in biochemical tests – the esculin-negative isolates were considered to be pathogenic. According to Wauters scheme (1987), the esculin-positive isolates belong to biotype 1A. The environmental isolates of biotype 1A are considered to be non-pathogenic because they lack the pYV plasmid and chromosomal virulence *ail* gene (Kraushaar *et al.*, 2011; Sihvonen *et al.,* 2012).

The majority of isolates isolated from food samples are not pathogenic. However, it is difficult to isolate pathogenic *Y. enterocolitica* that occur in low number in food samples and are accompanied by a high number of background flora. The application of RT-PCR enables to detect the pathogenic *Y. enterocolitica* in pig tonsils (Thisted and Lambertz, 2008). There are few well-determined chromosomal and plasmid-borne virulence factors present in pathogenic *Y. enterocolitica* and they are used as targets for RT-PCR (Falcao *et al.*, 2006; Wang *et al.*, 2010; Wang *et al.*, 2011). Expression of both chromosomal and plasmid genes is necessary to determine pathogenicity. One of the genes necessary to detect the virulence is *ail* gene present in pathogenic *Y. enterocolitica* (Lambertz *et al.*, 2008).

The detection rates of *ail*-positive *Y. enterocolitica* in pig tonsils were relatively low (Table II). The reason for the detection of numerous negative results could be low number of pathogenic strains present in pig tonsils (Fredriksson-Ahomaa and Korkeala, 2003). Another reason for the negative results could be background flora in the examined samples. Therefore, there is a necessity to carry out the selective enrichment step in order to eliminate the growth of background microflora because it decreases the sensitivity of the assay (van Damme, 2010). A good option is to use a selective enrichment medium CIN agar containing cefsulodin, irgasan and novobiocin which has been designed for RT-PCR detection of *Y. enterocolitica* (van Damme *et al.*, 2013a; 2013b). This medium contains selective components that inhibit the growth of background flora. The inclusivity and the exclusivity test show a potential in the applicability and reliability of the *ail* gene-based RT-PCR (Boyapalle *et al.*, 2001). The *ail* gene is chromosomally encoded and is inherited in comparison to the plasmid-encoded virulence factors that can be lost during the incubation because of instability of the virulence plasmid (Platt-Samoraj *et al.*, 2017).

The prevalence of this pathogen in naturally contaminated pig tonsils has previously been found to be high with RT-PCR. The results of this study showed that the presence of pathogenic *Y. enterocolitica* isolates in pigs slaughtered in the podlaskie voivodeship is relatively low. The microbiological contamination of carcasses pinpoints that good hygiene measures are required to control the spread of *Y. enterocolitica* isolates at abattoir especially during the evisceration and tonsils removal. Furthermore, the implementation of control programmes with the aim to reduce the number of carrier pigs at a farm level would be the best strategy to avoid the contamination at slaughterhouse. The culture method on CIN agar plates showed the growth of a high number of natural background microorganisms and it is not easy to distinguish presumptive pathogenic *Y. enterocolitica* from non-pathogenic ones. The biochemical tests are also not sensitive enough to detect the pathogenic isolates.

In summary, the effectiveness of detecting pathogenic *Y. enterocolitica* with PowerChek™ *Yersinia enterocolitica* RT-PCR Detection System has been described. The RT-PCR possesses a greater sensitivity in comparison to conventional methods for the detection of pathogenic *Y. enterocolitica* in animal and food samples. This method could find its application in meat processing plants as a reference method in the safety management system to monitor critical control points. Finding of two biotype 1A isolates possessing the *ail* gene widens the knowledge on the virulence of *Y. enterocolitica* biotype 1A. It means that some isolates of 1A biotype have also genes that are important in the pathogenesis of *Y. enterocolitica* infection.

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Literature

Bolton D.J., C. Ivory and D. McDowell. 2013. A small study of *Yersinia enterocolitica* in pigs from birth to carcass and characterization of porcine and human strains. *Food Control* 33: 521–524.

Bonardi S., I. Alpigiani, S. Pongolini, M. Morganti, S. Tagliabue, C. Bacci and F. Brindani. 2014. Detection, enumeration and characterization of *Yersinia enterocolitica* 4/O:3 in pig tonsils at slaughter in Northern Italy. *Int. J. Food Microbiol.* 177: 9–15.

Bottone E.J. 1997. *Yersinia enterocolitica*: the charisma continues. *Clin. Microbiol. Rev.* 10: 257–276.

Boyapalle S., I.V. Wesley, H.S. Hurd and P.G. Reddy. 2001. Comparison of culture, multiplex, and 5 nuclease polymerase chain reaction assay for the rapid detection of Yersinia enterocolitica in swine and pork products. *J. Food Protect.* 64: 1352–1361.

Falcao J.P., D.P. Falcao, A. Pitondo-Silva, A.C. Malaspina and M. Brocchi. 2006. Molecular typing and virulence markers of *Yersinia enterocolitica* strains from human, animal and food origins isolated between 1968 and 2000 in Brazil. *J. Med. Microbiol*. 55: 1539–1548.

Fredriksson-Ahomaa M. and H. Korkeala. 2003. Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: a methodological problem. *Clin. Microbiol. Rev.* 16(2): 220–229.

Kraushaar B., R. Dieckmann, M. Wittwer, D. Knabner, A. Konietzny, D. Made and E. Strauch. 2011. Characterization of a *Yersinia enterocolitica* biotype 1A strain harbouring an ail gene. *J. Appl. Microbiol.* 111: 997–1005.

Lambertz S.T., K. Granath, M. Fredriksson-Ahomaa, K.E. Johansson and M.L. Danielsson-Tham. 2007. Evaluation of a combined culture and PCR method (NMKL-163A) for detection of presumptive pathogenic *Yersinia enterocolitica* in pork products. *J. Food Prot.* 70(2): 335–340.

Lambertz S.T., C. Nilsson, S. Hallanvuo and M. Lindblad. 2008. Real-time PCR method for detection of pathogenic *Yersinia enterocolitica* in food. *Appl. Environ. Microbiol.* 74(19): 6060–6067.

Platt-Samoraj A., K. Syczyło, A. Szczerba-Turek, A. Bancerz-Kisiel, A. Jabłoński, S. Łabuć, J. Pajdak, N. Oshakbaeva and W. Szweda. 2017. Presence of *ail* and *yst*B genes in *Yersinia enterocolitica* biotype 1A isolates from game animals in Poland. *Vet. J.* 221: 11–13.

Sihvonen L.M., K. Jalkanen, E. Huovinen, S. Toivonen, J. Corander, M. Kuusi, M. Skurnik, A. Siitonen and K. Haukka. 2012. Clinical isolates of *Yersinia enterocolitica* Biotype 1A represent two phylogenetic lineages with differing pathogenicity-related properties. *BMC Microbiol.* 12: 208.

Tennant S.M., T.H. Grant and R.M. Robins-Browne. 2003. Pathogenicity of *Yersinia enterocolitica* biotype 1A. *FEMS Immunol. Med. Microbiol*. 38: 127–137.

Thoerner P., C.I. Bin Kingombe, K. Bogli-Stuber, B. Bissig--Choisat, T.M. Wassenaar, J. Frey and T. Jemmi. 2003. PCR detection of virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and investigation of virulence gene distribution. *Appl. Environ. Microbiol.* 69: 1810–1816.

Thisted Lambertz S., C. Nilsson, S. Hallanvuo and M. Lindblad. 2008. Real-time PCR method for detection of pathogenic *Yersinia enterocolitica* in food. *Appl. Environ. Microbiol.* 74: 6060–6067.

van Damme I., D. Berkvens, J. Baré and L. De Zutter. 2013a. Influence of isolation methods on the occurrence of plasmid-carrying *Yersinia enterocolitica* serotype O:3 in slaughter pig tonsils, faeces and carcass surface swabs. *Int. J. Food Microbiol.* 164: 325–327.

van Damme I., D. Berkvens, N. Botteldoorn, K. Dierick, J. Wits, B. Pochet and L. De Zutter. 2013b. Evaluation of the ISO 10273:2003 method for the isolation of human pathogenic *Yersinia enterocolitica* from pig carcasses and minced meat. *Food Microbiol.* 36: 170–175.

van Damme I., I. Habib and L. De Zutter. 2010. *Yersinia enterocolitica* in slaughter pig tonsils: Enumeration and detection by enrichment versus direct plating culture. *Food Microbiol.* 27: 158–161.

Wang X., Z. Cui, D. Jin, L. Tang, S. Xia, H. Wang, Y. Xiao, H. Qiu, Q. Hao, B. Kan, J. Xu and H. Jing. 2009. Distribution of pathogenic *Yersinia enterocolitica* in China. *Eur. J. Clin. Microbiol. Infect. Dis.* 28: 1237–1244.

Wang X., H. Qiu, D. Jin, Z. Cui, B. Kan, Y. Xiao, Y. Xu, S. Xia, H. Wang, J. Yang, W. Hu, J. Xu J and H. Jing. 2008. O:8 serotype *Yersinia enterocolitica* strains in China. *Int. J. Food Microbiol.* 125: 259–266.

Wang X., Z. Cui, H. Wang, L. Tang, J. Yang, L. Gu, D. Jin, L. Luo, H. Qiu, Y. Xiao, H. Xiong, B. Kan, J. Xu and H. Jing. 2010. Pathogenic strains of *Yersinia enterocolitica* isolated from domestic dogs (*Canis familiaris*) belonging to farmers are of the same subtype as pathogenic *Y. enterocolitica* strains isolated from humans and may be a source of human infection in Jiangsu Province, *China. J. Clin. Microbiol*. 48: 1604–1610.

Wang X., Y. Li, H. Jing, Y. Ren, Z. Zhou, S. Wang, B. Kan, J. Xu and L. Wang. 2011. Complete genome sequence of a *Yersinia enterocolitica* "Old World" (3/O:9) strain and comparison with the "New World" (1B/O:8) strain. *J. Clin. Microbiol*. 49: 1251–1259.

Wauters G., K. Kandolo and M. Janssens. 1987. Revised biogrouping scheme of *Yersinia enterocolitica*. *Contrib. Microbiol. Immunol*. 9: 14–21.

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SHORT COMMUNICATION

Global Transcriptome Changes of Biofilm-Forming *Staphylococcus epidermidis* Responding to Total Alkaloids of *Sophorea alopecuroides*

CUI-PING GUAN, HUI-XIA LUO, FANG HE and XUE-ZHANG ZHOU*

Key Laboratory of the Ministry of Education for the Conservation and Utilization of Special Biological Resources in Western China, Ningxia University, Yinchuan, Ningxia, China

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Abstract

Transcriptome changes of biofilm-forming *Staphylococcus epidermidis* response to total alkaloids of *Sophorea alopecuroides* was observed. Bioinformatic analyses were further used to compare the differential gene expression between control and the treated samples. It was found that 282 genes were differentially expressed, with 92 up-regulated and 190 down-regulated. These involved down-regulation of the sulfur metabolism pathway. It was suggested that inhibitory effects on *Staphylococcus epidermidis* and its biofilm formation of the total alkaloids of *S. alopecuroides* was mainly due to the regulation of the sulfur metabolism pathways of *S. epidermidis*.

Key words: *Staphylococcus epidermidis*, sulfur metabolism, total alkaloids of *Sophorea alopecuroides*, transcriptome

S. epidermidis is an opportunistic pathogen; however, it is capable of storing and transmitting drug-resistant genes (Bloemendaal *et al.*, 2010; Otto, 2013). The ability of *S. epidermidis* to form biofilms *in vivo* allows it to be highly resistant to chemotherapeutics and can ultimately lead to chronic disease (Cerca *et al.*, 2005). Some Chinese medicinal herbal products have been found to prevent biofilm formation in bacteria (Guan *et al.*, 2013; Wang *et al.*, 2015; Yang *et al.*, 2016). Total alkaloids of *S. alopecuroides* (TASA) are an alkaloid mixture that has demonstrated a better inhibitory effect on the late stage of *S. epidermidis* biofilm thickening than ciprofloxacin (CIP), and erythromycin (ERY) (Li *et al.*, 2016).

In order to investigate the inhibitory mechanism of TASA, the global transcriptome changes of biofilm-forming *S. epidermidis* (ATCC 35984) in response to TASA were analysed in this study. Some pre-experiments were performed to determine the transcriptome sequencing conditions. In the pre-experiments, the relative expression quantity of four main biofilm formation related genes (*ica*, *sigB*, *agr* and *fbe*) were compared after the *S. epidermidis* treatment by TASA in different sub-MIC (1/2 MIC and 1/4 MIC) and time periods (6 h, 12 h, and 24 h). The results showed that these four genes were down-regulated significantly when the *S. epidermidis* was treated by 1/2 MIC (12.5 mg/ml) of TASA for 12 h. Based on the pilot experiment results, the transcriptome sequencing was used to investigate the transcriptional differences of *S. epidermidis* ATCC 35984 in a blank-medium control group and the treatment group, cultured with 1/2 MIC of TASA for 12 h. The inhibitory mechanism of Chinese medicine TASA was examined through the bioinformatic analyses.

S. epidermidis ATCC 35984 was cultured overnight at 37°C, 120 rpm. The resulting suspension was diluted to 0.5 McFarland with TSB medium and 3 ml of the diluted suspension was seeded into a six-well plate. The plate was sealed with an aseptic glass cover and placed in the incubator upside down. After 12 h of incubation at 37°C, the liquid in each well was removed and washed twice with PBS. The biofilm bacteria adhered to the glass cover in the blank control samples were collected with 1 ml TSB, and centrifuged at 12000 r/min for 10 min at 4°C. For TASA-treated samples, the biofilm bacteria on the glass covers were incubated for an additional 12 h in the presence of TASA at 12.5 mg/ml (1/2 MIC) prior to the bacteria harvesting, which followed the same methodology as the blank control. Total mRNA extraction was performed with RNAprep pure Cell/Bacteria Kit (TianGen), according to the manufacturer's instructions, then stored at –80°C, and prepared for transcriptome sequencing.

Standard sequencing procedure and data analysis were performed by using Illumina HiSeq 2500 high-throughput sequencing platform from Biomarker Technologies Co., Ltd (China). Separate sequence read

* Corresponding author: X.Z. Zhou, Department of College of Life Science, Ningxia University, Yinchuan, Ningxia, China; **e-mail: zhouxuezhang@nxu.edu.cn**

dataset was used as inputs into DESeq2 package (Love *et al.*, 2014) to analysis the unigenes expression based on RPKM (reads per kilobase transcriptome per million mapped reads). A fold change of ≥ 2 and a minimum false discovery rate (FDR) of < 0.01 were accepted as indicators of the differentially expressed genes (DEGs) after Benjamini-Hochberg *post hoc* correction. The DEGs were BLASTX against the Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Group (COG) and Gene Ontology (GO) to identify their predicted biological function. In order to verify the RNA-Seq results, the expression levels of selected DEGs were quantified by RT-qPCR. First, Nanodrop 2000 (Thermo Scientific, Wilmington, USA) was used to measure the concentration of total mRNA and 500 ng of RNA was reverse-transcribed into cDNA with PrimeScript™ RT Master Mix (TaKaRa). RT-qPCR was performed with an iQ5 light cycler (Bio-Rad) by using SYBR® Premix EX Taq™ II (TaKaRa) in a 20 µl reaction volume, which consisted of 0.25 mM of each primer, 10 µl of SYBR Premix Ex Taq II and 1 µL of template cDNA. PCR conditions were as follows: 30 s at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 58°C. The reference gene was 16S rRNA, data were acquired through Bio-Rad and analyzed by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All experiments were performed in triplicate.

282 unigenes were differentially expressed under the screening criteria (fold change ≥ 2 and FDR < 0.01). Within these DEGs, 92 unigenes were significantly up-regulated and 190 unigenes were significantly down-regulated. The DEGs were searched against the KEGG database to identify their biological pathways. The 20 greatest enriched pathways were listed in Fig. 1. Pathways (represented by symbols) in the upper left quadrant of Fig. 1 contain DEGs with more significant and reliable enrichment levels, therefore the sulfur metabolism pathway ranked first. In sulfur metabolism pathway, there are nine DEGs which were all down-regulated significantly, corresponding to the encoded enzymes included serine acetyltransferase (CysE, EC 2.3.1.30), phosphoadenosine phosphosulfate reductase (CysH, EC 1.8.1.2), sulfite reductase (CysI and CysJ, EC 1.8.4.8), cystathionine beta-lyase (Cbl, EC 2.5.1.48), cysteine synthase (CysK and CysM, EC 2.5.1.47), sulfate adenylyl transferase (Sat, EC 2.7.7.4) and adenylylsulfate kinase (CysC, EC 2.7.1.25) (Fig. 2). These enzymes directly affect the content of sulfur metabolites such as cysteine (Cys), methionine (Met), glutathione (GSH), etc. RT-qPCR results further demonstrated that all nine genes showed similar expression patterns to those of

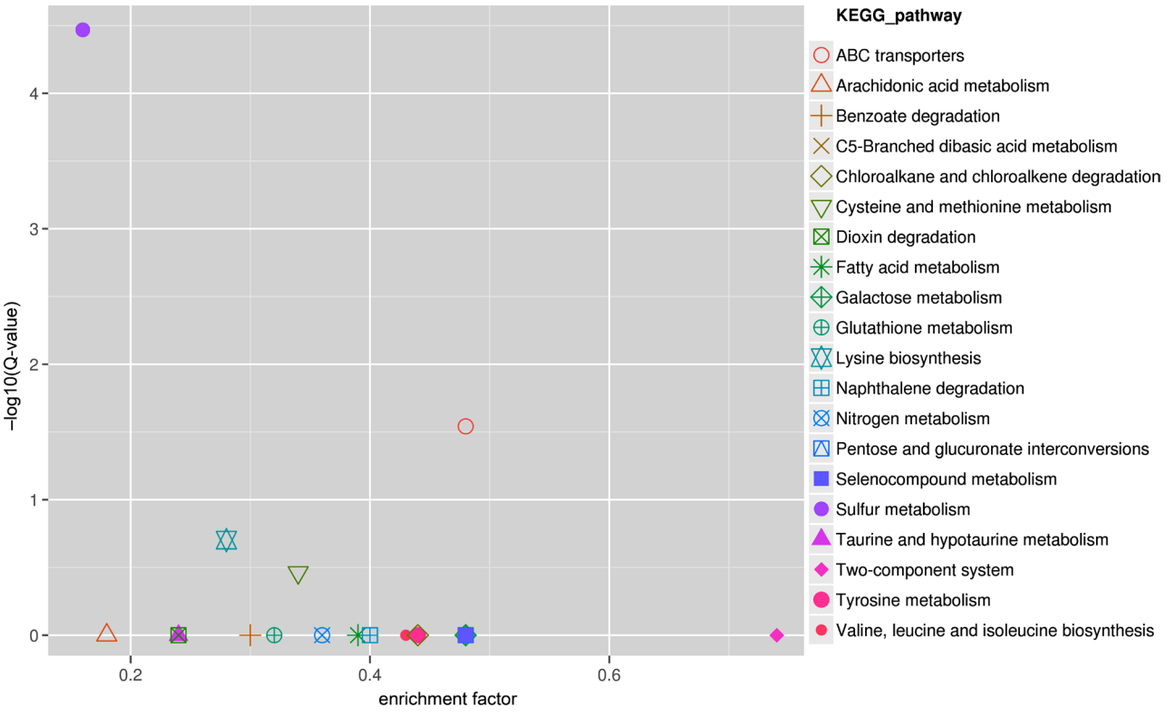

Fig. 1. KEGG pathway scatter diagram of differentially expressed genes. Each pattern represents a KEGG pathway with its name listed in the right. X-coordinate represents the enrichment factor showing the ratio of all genes annotated in the pathway, in contrast to the differentially expressed genes annotated in the same pathway. The smaller the enrichment factor, the enrichment level of the differentially expressed genes was more significant in this pathway. Y-coordinate represents log10(Q_value). The higher the absolute Q_value, the enrichment level of the differentially expressed genes was more significant and reliable in this pathway.

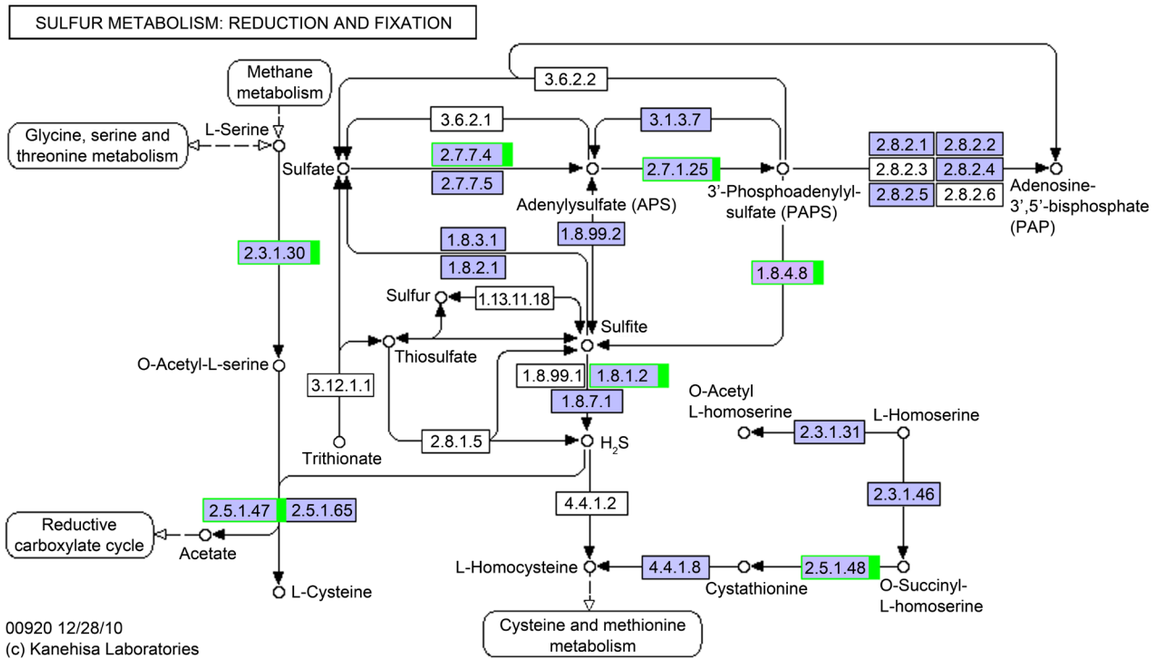

Fig. 2. Distribution of the differentially expressed genes in a sulfur metabolism pathway. Compared to the control group, the enzyme marked by a green frame is related to a drown-regulated gene product. The number in the frame represents the enzyme code.

RNA-seq, confirming the RNA-seq based transcriptome datasets were accurate and robust (Fig. 3).

In this research, according to the analysis of transcriptome data, the sulfur metabolism pathway in *S. epidermidis* was greatly influenced by TASA. Sulfur metabolism is an important metabolic pathway in bacteria and its metabolites are involved in many physiological and biochemical processes in cells (Zeng *et al.*, 2013). Cysteine (Cys), methionine (Met) and glutathione (GSH) are associated with bacterial activity and biofilm formation (Murillo *et al.*, 2005; Gales *et al.*, 2008; Soutourina *et al.*, 2009; Li *et al.*, 2013; Solis *et al.*, 2016). A link between sulfate assimilation, Met biosynthesis and biofilm formation have been found in *Candida albicans* (Murillo *et al.*, 2005). Deletion of the *ecm17* gene encoding the sulfite reductase beta subunit

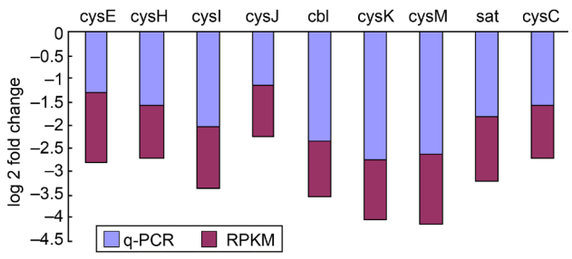

Fig. 3. Comparison of folds changes detected by RNA-seq (RPKM) and q-PCR.

Bars represent the log2 value of the fold changes of the gene expressions between the control and TASA treatment groups. On the top of the bars are the abbreviations of enzymes encoded by the nine genes.

resulted in reduced adhesion and poor biofilm formation in *C. albicans* (Li *et al.*, 2013). Additionally, deletion of the master regulator of Cys metabolism *cymR* in *S. aureus* also results in diminished biofilm formation (Soutourina *et al.*, 2009). A recent research has provided further evidence of a role for sulfate assimilation and Cys/Met biosynthesis in *S. epidermidis* ATCC 35984 (RP62A) biofilm formation (Solis *et al.*, 2016). According to the comparison of non-biofilm forming *S. epidermidis* ATCC 12228 and biofilm-forming ATCC 35984, Solis *et al.* (2016) showed that the sulfate assimilation and cysteine/methionine biosynthesis pathways in ATCC 35984 contained elevated levels (~25% increase) of methionine that were likely linked to biofilm formation. GSH, another important sulfur metabolite, which is associated with intracellular reactive oxygen species (ROS) and hydrogen sulfide (H_2S) also played an important role in biofilm formation (Gales *et al.*, 2008; Klare *et al.*, 2016; Ooi and Tan, 2016). In this study, TASA significantly influenced the sulfur metabolism by down-regulation of nine important genes in this pathway. The concentration of Cys, Met and GSH were directly affected, and most probably finally disrupted the biofilm formation of *S. epidermidis*. This result also validated the above-mentioned relationship between sulfur metabolism and biofilms.

In the pre-experiments, the relative expression of biofilm-related genes including *ica*, *sigB*, *agr* and *fbe* in *S. epidermidis* was measured. Results showed that these four genes were differentially expressed after treatment

with TASA at 1/2 MIC for 12 h. However, these four genes did not appear in the 282 DEGs obtained by transcriptome sequencing due to the DEGs screening criteria (fold change ≥ 2 and FDR < 0.01). It was suggested that genes related to biofilm formation did have differential expression, but they may not be directly regulated by TASA. Instead, their expression may be indirectly affected by the changes of other metabolic pathways (*i.e.* sulfur metabolism).

For a long time, the formation of biofilm has been widely recognized as a dynamic process and regulated by some genes including *ica*, *sigB*, *agr* and *fbe etc.* Now, it was shown that there is a relationship between sulfur metabolism and biofilm formation in *S. epidermidis*. Based on the results of transcriptome analysis, it was suggested that TASA's inhibitory effects on *S. epidermidis* and its biofilm formation is mainly due to the regulation of the sulfur metabolism pathway.

The regulatory mechanism of TASA has not been analyzed due to its multi-channeled and multi-targeted actions. In the study, the effect of TASA on *S. epidermidis* was comprehensively analyzed by using RNA-seq. The enrichment of DEGs showed that metabolism, genetic information processing and environmental information processing were greatly influenced by TASA. The sulfur metabolism pathway was the most significant with all of the key enzymes in this pathway being down-regulated. Given the important physiological role of sulfur metabolism in bacteria and its effect on biofilm formation, it is concluded that the inhibitory effect of TASA on *S. epidermidis* and its biofilm formation are mainly due to its actions on this pathway.

In conclusion, according to the analysis of differential expression and metabolic pathway enrichment, the molecular mechanism of TASA regulation of pathogenicity, virulence and metabolism of *S. epidermidis* was further characterized. The sulfur metabolism pathway was identified and results could provide valuable information for follow-up studies to examine the regulatory mechanism of TASA on *S. epidermidis*, and could serve as the basis for exploring potential drug targets.

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Literature

Bloemendaal A.L., E.C. Brouwer and A.C. Fluit. 2010. Methicillin resistance transfer from *Staphylocccus epidermidis* to methicillin-susceptible *Staphylococcus aureus* in a patient during antibiotic therapy. *PloS One* 5(7): e11841.

Cerca N., S. Martins, F. Cerca, K.K. Jefferson, G.B. Pier, R. Oliveira and J. Azeredo. 2005. Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. *J. Antimicrob. Chemother.* 56(2): 331–336.

Gales G., M. Penninckx, J.C. Block and P. Leroy. 2008. Role of glutathione metabolism status in the definition of some cellular parameters and oxidative stress tolerance of Saccharomyces cerevisiae cells growing as biofilms. *FEMS Yeast Res.* 8(5): 667–675.

Guan Y., C. Li, J.J. Shi, H.N. Zhou, L. Liu, Y. Wang and Y.P. Pu. 2013. Effect of combination of sub-MIC sodium houttuyfonate and erythromycin on biofilm of *Staphylococcus epidermidis. Zhongguo Zhong Yao Za Zhi* 38(5): 731–735.

Klare W., T. Das, A. Ibugo, E. Buckle, M. Manefield and J. Manos. 2016. Glutathione-disrupted biofilms of clinical *pseudomonas aeruginosa* strains exhibit an enhanced antibiotic effect and a novel biofilm transcriptome. *Antimicrob. Agents Ch.* 60(8): 4539–4551.

Livak K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25(4): 402–408.

Li D., Y. Wang, B. Dai, X. Li, L. Zhao, Y. Cao, L. Yan and Y. Jiang. 2013. ECM17-dependent methionine/cysteine biosynthesis contributes to biofilm formation in *Candida albicans*. *Fungal Genet. Biol.* 51: 50–59.

Li X., C.P. Guan, Y.L. He, Y.J. Wang, X.M. Liu and X.Z. Zhou. 2016. Effects of total alkaloids of *Sophorea Alopecuroides* on biofilm formation in *Staphylococcus epidermidis. Biomed. Res. Int.* 2016: 4020715.

Love M.I., W. Huber and S. Anders. 2014. Moderate destimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 15: 550.

Murillo L.A., G. Newport, C. Lan, S. Habelitz, J. Dungan and N.M. Agabian. 2005. Genome-wide transcription profiling of the early phase of biofilm formation by *Candida albicans*. *Eukaryot. Cell* 4(9): 1562–1573.

Ooi X.J. and K.S. Tan. 2016. Reduced glutathione mediates resistance to H_2S toxicity in *oral Streptococci*. *Appl. Environ. Microb.* 82(7): 2078–2085.

Otto M. 2013. Coagulase-negative staphylococci as reservoirs of genes facilitating MRSA infection: Staphylococcal commensal species such as *Staphylococcus epidermidis* are being recognized as important sources of genes promoting MRSA colonization and virulence. *Bioessays* 35(1): 4–11.

Solis N., J.A. Cain and S.J. Cordwell. 2016. Comparative analysis of *Staphylococcus epidermidis* strains utilizing quantitative and cell surface shaving proteomics. *J. Proteomics* 130: 190–199.

Soutourina O., O. Poupel, J.Y. Coppée, A. Danchin, T. Msadek and I. Martin-Verstraete. 2009. CymR, the master regulator of cysteine metabolism in Staphylococcus aureus, controls host sulphur source utilization and plays a role in biofilm formation. *Mol. Microbiol.* 73(2): 194–211.

Wang T., G. Shi, J. Shao, D. Wu, Y. Yan, M. Zhang, Y. Cui and C. Wang. 2015. In vitro antifungal activity of baicalin against *Candida albicans* biofilms via apoptotic induction. *Microb. Pathog.* 87: 21–29.

Yang Y., B.I. Park, E.H. Hwang and Y.O. You. 2016. Composition analysis and inhibitory effect of *Sterculia lychnophora* against biofilm formation by *Streptococcus mutans*. *Evid. Based Complement Alternat. Med*. 2016: 8163150.

Zeng L.R., T.Y. Shi, Q.J. Zhao and J.P. Xie. 2013. *Mycobacterium* sulfur metabolism and implications for novel drug targets. *Cell Biochem. Biophys.* 65(2): 77–83.

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SHORT COMMUNICATION

Sero-epidemiology and Risk Factor Analysis of Measles Among Children in Pakistan

MUHAMMAD HIDAYAT RASOOL[1]*, AHSAN RAFIQ[1], MUHAMMAD ZEESHAN NAWAZ[1], MUHAMMAD SHAFIQUE[1] and MUHAMMAD SAQALEIN[1]

[1] Department of Microbiology, Government College University, Faisalabad, Pakistan

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Abstract

Comparative cross sectional study was conducted on blood samples (n = 231) collected from children of 1 to 10 years of age in Punjab Pakistan through convenient sampling method. Indirect haemagglutination assay (IHA) was standardized and used for serodiagnosis and evaluation of humoral immunity against measles. Associated risk factors including age, gender, locale, and vaccination status were analyzed. Geometric mean titre (GMT) of vaccinated individuals was significantly higher ($p < 0.001$) than that of non-vaccinated individuals showing that IHA titre of vaccinated individuals was a measure of humoral immune response; whereas, in case of non-vaccinated individuals an indicative of exposure to the measles infection.

Key words: measles, sero-epidemiology, geometric mean titre, IHA

Measles, a disease caused by Measles virus of genus Morbillivirus is a highly contagious disease, which is transmitted through respiratory droplets of infected person (Rasool *et al.*, 2016). Measles virus possess H-protein for it attachment to the target site, which is the main cause of this disease. The genome of Measles virus is composed of non-segmented single stranded RNA, which encodes for different proteins (Griffin *et al.*, 2007). Before the availability of vaccine against measles, about 2.6 million deaths have been reported annually due to this extremely hazardous disease (Perry and Halsey, 2004). Globally, measles was a main reason of early childhood mortality because no efficient and cost-effective vaccine was available against it. The measles prevalence rates are more among children above 12 months of age with high severity in children with vitamin A deficiencies (Merajuddin *et al.*, 2015).

Recently, several disastrous epidemics have been exploded in different areas of the world including Europe and China. About 1.5 million deaths were reported in 2010 only. According to a report of WHO in 2012, about 1.2 million individuals were died of measles and majority of them were young children up to 5 years of age. According to an estimate, the case fatality ratios (CFRs) of measles are about 0.1% in developed countries and up to 30% in immigrant people (Perry and Halsey, 2004). During the time period of 1999–2005, measles mortality and morbidity rate has been reduced up to 60%; however, yet causalities due to measles are still far above the ground in various parts of the world (Cohen *et al.*, 2009).

Measles can lead to many severe complications including pneumonia, encephalitis and even death. After infection, contagious encephalitis may also develop about 1/1,000 registered measles patients and mortality rate is about 2–3 deaths/1,000 measles cases (Gindler *et al.*, 2004). A considerable reduction in prevalence, morbidity and mortality from measles may be achieved by proper immunization coverage. In Pakistan, the usual immunization coverage for measles remains < 60% (Zahoor *et al.*, 2015). The major factors for low vaccination coverage include the lack of education and lack of motivation. In non-vaccinated individuals, the risk of measles complications is very high and these complications can only be reduced by proper vaccination (Mohammad *et al.*, 2011).

Several studies recommend that the routine vaccination program is the only way to achieve high level of immunity in the community (Shakurnia *et al.*, 2013). Generally in Pakistan, the vaccination exposure against vaccine treatable infections varied from 56% to 88% among various populations of different provinces in

* Corresponding author: M.H. Rasool, Department of Microbiology, Government College University, Faisalabad, Pakistan, **e-mail: drmhrasool@gcuf.edu.pk**

2011 and 2012. A large figure of measles epidemics with high morbidity and mortality rates has been recorded in various regions of Pakistan (Merajuddin *et al.*, 2015). In Pakistan, while considering the measles vaccination approach, measles epidemics, poor vaccination knowledge and lack of vaccination services in distant and countryside regions, the various society dependant studies have been carried out in different regions of Pakistan to assess the measles incidence in infants in spite of immunization (Merajuddin *et al.*, 2015).

The significance of an early and accurate diagnosis cannot be overlooked to adopt the effective control measures against a disease (Moss and Strebel, 2011). Clinicians can perform these EIAs very easily with only single sample of minute quantity and carried out after 4 weeks of manifestation of rash (Ratnam *et al.*, 2003). The diagnosis of measles can be done through different ways now a day. Initially, in early days only clinical diagnosis was done without laboratory confirmation. The laboratory diagnosis was carried out by applying usual techniques such as haemolysin inhibition, haemagglutination inhibition (HI), complement fixation, and plaque-reduction neutralization (PRN) for determination of antibodies against measles while immunefluorescence antibodies technique was used for recognition of measles (Featherstone *et al.*, 2011).

Antibodies against Measles can also be detected using indirect haemagglutination assay (IHA). IHA is found to be simple, specific and cost effective therefore, it was chosen for the assessment of serum samples in present study. After optimization of test conditions, IHA was used for the evaluation of humoral immune response against measles and for assessment of certain risk factors associated with the disease in District Sargodha and Khushab of Punjab Pakistan.

Children of 1 to 10 years of age from different areas of district Sargodha and Khushab, Punjab Pakistan were selected as target population. They were further divided and assessed on the basis of different parameters *i.e.* gender (male and female), area (urban, peri-urban and rural), age (1–3 years, 4–6 years and 7–10 years) and vaccination status (vaccinated or non-vaccinated). A total of 231 serum samples (134 from Sargodha and 97 from Khushab) were randomly collected from children ranging from 1 to 10 years of age. 138 serum samples from male and 93 samples from female children were collected. Similarly, 139 serum samples were from vaccinated children of both sexes and 92 samples from non-vaccinated ones. Complete record of collected samples was maintained on data sheet containing information like individual's name, age, locale, gender and vaccination status. The prior permission for blood sampling was obtained from Ethical Review Committee of Government College University Faisalabad. After collection, blood samples were properly labelled and stored in Gel & Clot Activator Vacutainers®. These Samples were then, centrifuged at 3000 rpm for 5 minutes for collection of serum. The collected sera were labelled and kept at –20°C till further processing. Serum samples were heat inactivated at 56°C for about 30 minutes in water bath to inactivate the complement proteins for preventing non-specific hemolysis (Soltis *et al.*, 1979). Blood samples were collected from sheep, chicken, and rabbit and centrifugation of these blood samples was carried out at 1500 rpm for 5 minutes to get RBCs. Pelleted RBCs were washed using phosphate buffer saline (PBS) and 10% stock suspension of erythrocytes was prepared from washed RBCs of sheep, chicken and rabbit using PBS for optimization of test conditions. The erythrocytes were fixed with 0.1% gluteraldehyde (Gluteraldehyde 25% Applichem™) and tanned with tannic acid (10 mg/dl) for about 30 minutes at 37°C (Iwasa *et al.*, 1977). Live attenuated (freeze dried) Measles vaccine (Indonesia) was obtained from Health Department, Government of the Punjab and used as antigen. After ultrasonication (Donald *et al.*, 1967) of antigen, sensitization of RBCs was done by treatment with measles antigen for a 1 hour at 37°C. 1% and 2% suspension of sensitized RBCs of sheep, chicken and rabbit were prepared. The optimized test conditions showing best and reproducible results were used for testing of serum samples.

Indirect haemagglutination antibodies (IHA) titers of all the serum samples were measured against measles virus antigens by applying the technique explained by Sakata and Sugiura (1988). The test was performed using micro-titration plates each comprising of 96 U-shaped wells. The highest dilution of each serum sample showing a clear haemagglutination pattern was considered as end point and taken as positive whereas button formation as negative. The IHA antibodies titre was exhibited as the reciprocal of its end point dilution. The IHA titers of all the serum samples thus obtained were recorded and Geometric mean titers (GMTs) were calculated and analyzed on the basis of gender, locale, age groups and vaccination status. The data was analyzed statistically by independent t-test and a one way Analysis of Variance (ANOVA). A value of $p \leq 0.05$ was considered as significant (Ruzauskas, 2005).

Indirect Haemagglutination Assay was optimized using gluteraldehyde (0.1%) and tannic acid (10 mg/dl) with RBCs obtained from different species including sheep, chicken and rabbit. Following optimization of test conditions, it was used for the sero-diagnosis and evaluation of humoral immune response against measles in Sargodha and Khushab districts of Punjab, Pakistan. 2% sheep erythrocyte suspension fixed with 0.1% gluteraldehyde and tanned with tannic acid (10 mg/dl), and adsorbed with ultra-sonicated measles antigen showed the clear and reproducible results in

terms of agglutination and button formation as compared to 1% sheep RBCs.

IHA antibodies titre of 16 or more was considered as positive and < 8 as negative. Similarly, GMT value of 8 was also taken as negative. A total of 231 serum samples from district Sargodha (n = 134) and Khushab (n = 97) were collected from urban, peri-urban and rural areas and processed through IHA. The GMT of all 231 samples of district Sargodha and Khushab showed non-significant ($p > 0.05$) difference among males and females. The mean GMT of male was 77.51; while that of female was 66.15.

Comparison of GMTs of vaccinated and non-vaccinated children of both districts revealed a highly significant ($p < 0.01$) difference. The GMT of vaccinated individuals was higher than that of non-vaccinated individuals. Similarly, a highly significant ($p < 0.01$) difference was found in antibody titre among individuals of urban, peri-urban and rural areas. The mean antibody titre of individuals of urban area was highest when compared to individuals from peri-urban and rural areas. There was also a significant difference in the antibody titre of individuals of district Sargodha and Khushab. The mean antibody titre of individuals of district Khushab was high than that of district Sargodha. The samples obtained from both districts were divided into 3 age groups *i.e.*, 1–3 years, 4–6 years and 7–10 years. A significant difference in the antibody titre of individuals in these age groups was observed. The mean antibody titre of individuals of age 7–10 years was higher than that of other two age groups (Table I).

The GMT of 231 samples of district Sargodha was compared and there was non-significant difference in the results of GMT of both males and females of district Sargodha. The GMT of male was 56.54; whereas that of female was 52.00. GMT of individuals of district Sargodha was compared on the basis of different area like urban, peri-urban and rural. The results revealed that there was a highly significant difference in the antibody titre of individuals in urban, peri-urban and rural areas. The GMT of individuals of urban area was higher than that of peri-urban and rural areas (Table II).

The GMTs of 97 samples of district Khushab was compared and results revealed that there was non-significant difference among males and females of district Khushab. The mean GMT of male was 104.77; while that of female was 87.57. GMTs of vaccinated and non-vaccinated individuals of district Khushab revealed a highly significant difference. The GMT of vaccinated individuals was higher than that of non-vaccinated individuals. In district Khushab, GMT of individuals of urban area was higher than that of peri-urban and rural areas. Similarly, GMT in individuals of age 7–10 years was higher than that of other two age groups (Table III).

Table I
Overall comparison of GMTs of both districts on the basis of different parameters.

Geometric Mean Titre (GMT) and P-values											
Gender		Vaccination status		Age groups (Years)			Locale			District	
Male	Female	Yes	No	1–3	4–6	7–10	Urban	Peri-urban	Rural	Sargodha	Khushab
77.51	66.15	103.65	26.52	21.90	61.96	105.03	109.42	66.56	43.56	54.64	98.21
0.660^{NS}	0.704^{NS}	4.675	5.673	0.001**			0.007**			–2.580	–2.399

Table II
Comparison of GMTs on the basis of different parameters in district Sargodha.

Geometric Mean Titre (GMT) and P-values									
Gender		Vaccination status		Age groups (Years)			Locale		
Male	Female	Yes	No	1–3	4–6	7–10	Urban	Peri-urban	Rural
56.54	52.00	71.72	24.04	15.86	54.27	72.72	100.00	49.09	21.95
0.265^{NS}	0.276^{NS}	2.783	3.628	0.037*			0.001**		

Table III
Comparison of GMTs on the basis of different parameters in district Khushab.

Geometric Mean Titre (GMT) and P-values									
Gender		Vaccination status		Age groups (Years)			Locale		
Male	Female	Yes	No	1–3	4–6	7–10	Urban	Peri-urban	Rural
104.77	87.57	155.47	29.23	34.92	69.17	159.78	146.00	96.52	42.60
0.517^{NS}	0.562^{NS}	4.239	4.634	0.009**			0.029*		

In present study, standardization and optimization of IHA was carried out to assess humoral immune response to measles among children because measles is a highly infectious disease and one of the major reasons of mortality and morbidity among children in the whole world, mainly in developing countries. In urbanized countries, especially Europe and US, measles infection has been controlled through immunization. However, still developing countries are being affected by measles due to inadequate vaccine exposure and inappropriate management of vaccines (Merajuddin *et al.*, 2015). Although vaccine is available for the measles yet it is a major disease in developing and third world countries because of high cost of available vaccine and mishandling of vaccine due to inappropriate storage facilities (Mehnaz, 2011). In Pakistan, vaccination has constantly been under achieved. The WHO reported data reveals that in 2010, usually planned vaccination coverage was 68% with Punjab (86%), Khyber Pakhtunkhwa (74%), Sindh (68%) and a very less proportion was recorded from Baluchistan (43%) (Khan and Qazi, 2014). According to a cross sectional study in 2015, the overall incidence of antibodies against measles was about 93.5% in the population of Faisalabad, Punjab (Rasool *et al.*, 2016).

Measles epidemics were and are still incident in various regions of the world with large ratio of reported cases and mortalities in a short duration of time. This huge gap is due to different reasons including duplicity in healthcare system, poor healthcare facilities, lower immunization coverage, undermined usual immunization, negligence among parents, and lack in quantity of vaccinators (Niazi and Sadaf, 2014). The efficiency of measles vaccine and improvement of immunity against measles among individuals of early age was not most favourable according to WHO strategies and un-vaccinated individuals were at high risk of measles infection (Zahoor *et al.*, 2015). For positive estimation of antibodies level of alleged cases of measles, highly sensitive serological tests are necessary (WHO, 2008).

Considering the significance of this infection particularly in the circumstances of present epidemics, this study was carried out with the aim of optimization of Indirect Hemagglutination Assay for the evaluation of humoral immune response against measles in district Sargodha and Khushab. Erythrocytes of various species including sheep, chicken and rabbit were used to perform IHA (Rasool *et al.*, 2016). In the past, excellent results were obtained when gluteraldehyde treated and sensitized RBCs of monkey were used in IHA (Gykha *et al.*, 1973). Because monkeys are not easily accessible so IHA was carried out with RBCs of sheep, rabbit and chicken in present study. 2% concentration of sheep RBC's revealed clear and reproducible results in this study however 0.5% and 1% erythrocytes concentrations have also been used in the past (Sakata and Suguira, 1988).

In this study, after optimization and standardization of IHA with 2% sheep RBC's fixed with 0.1% gluteraldehyde and adsorbed with measles antigen, IHA titres were calculated from the sera samples (n = 231) collected from children of district Sargodha and Khushab to assess the immunity against measles. The results of this study suggested that there was greater variation in the GMTs of vaccinated and non-vaccinated children of both districts *i.e.* Sargodha and Khushab. Non-significant difference in the GMTs values of males and females of both districts was observed demonstrating that measles infection is not dependant on gender (Ogundiji *et al.*, 2013). Statistical analysis of GMTs of different areas (urban, peri-urban and rural) of both districts showed significant differences in values indicating that less developed areas have more chances of measles infection due to low vaccination coverage, poor sanitary conditions, and lack of awareness in parents about the disease. The GMTs of individuals of age group 7–10 years were higher than other two groups indicating that immunity has been developed in elder children with the passage of time, while early age individuals have more chances of developing the disease.

In conclusion, it was accomplished that IHA with 2% sensitized sheep erythrocytes provided the most clear, consistent and reproducible results as compared to 1% sensitized sheep RBCs. Furthermore, it was found to be an inexpensive and valuable sero-diagnostic tool and hence can be used for the evaluation of humoral immune response against measles among different populations. Furthermore low antibody titers in non-vaccinated children indicate their susceptibility to measles infection with wild type of virus. So there is an urgent need for mass scale vaccination keeping in view the international heath standards to save our future generations against this devastating disease.

Literature

Cohen A.L., A. Salam, A. Bosan, R. Perry, S. Iqbal, S.N. Qureshi and R. Hafiz. 2009. Etiology of a suspected measles outbreak: preceding measles reduction activities in Pakistan. *J. College of Phys. Surg. Pak.* 19: 591–594.

Donald G., J.G. Martin and C. Kelsey. 1967. Physicochemical and biological properties of sonically treated Vi antigen. *J. Bacteriol.* 94(5): 1411.

Featherstone D.A., P.A. Rota, J. Icenogle, M.N. Mulders, Y. Jee and H. Ahmed. 2011. Expansion of the global measles and rubella laboratory network 2005–2009. *J. Infect. Dis. Suppl.* 1: 491–498.

Ghyka G.R., C. Cernescu and N. Cajal. 1973. Studies on the sensitivity of a passive hemagglutination test in the detection of anti-measles antibodies. *Revue Roumaine de Virologie.* 10: 295–300.

Gindler J., S. Tinker and L. Markowitz. 2004. Acute measles mortality in the United States. *J. Infect. Dis.* Suppl. 1. 189: 69–77.

Griffin D.E., M.A. Riddell, W.J. Moss, D. Hauer and M. Monze. 2007. Slow clearance of measles virus RNA after acute infection. *J. Clin. Virol.* 39: 312–317.

Helfand R.F., J.L. Heath, L.J. Anderson, E.F. Maes, D. Guris and W.J. Bellini. 1997. Diagnosis of measles with an IgM capture EIA: the optimal timing of specimen collection after rash onset. *J. Infect. Dis.* 175: 195–199.

Iwasa S., I. Yoshida, H. Ito and M. Hori. 1977. Application of freeze-dried monkey erythrocytes to measles viral hemagglutination and hemagglutination-inhibition tests. *J. Clin. Microbiol.* 6(2): 176.

Khan T. and J. Qazi. 2014. Measles outbreaks in Pakistan: causes of the tragedy and future implications. *Epidemiol. Reports* 2(1): 9911–2054.

Mehnaz A. 2009. Infectious diseases in children – still leads. *J. Pak. Med. Assoc.* 59 (7): 425–426.

Merajuddin K., A. Obaid Ullah, A. Ambreen and I. Ahmad. 2015. Measles in vaccinated children 1.5 to 3 years of age in rural community of district Peshawar, Pakistan. *J. Ayub Med. Coll. Abbottabad.* 27(4): 7–10.

Mohammad A., M. Irshad and B. Khan. 2011. A comparative study of measles complications in vaccinated versus non-vaccinated children. *J. Post. Med. Inst.* 25(1): 4–8.

Moss W.J. and P. Strebel. 2012. Biological feasibility of measles eradication. *J. Infect. Dis.* 204(1): 47–53.

Neumann P.W., J. Weber, A.G. Jessamine and M.V. O'Shaughnessy. 1985. Comparison of measles antihemolysin test, enzyme-linked Immunosorbant assay, and hemagglutination inhibition test with neutralization test for determination of immune status. *J. Clin. Microbiol.* 22: 296–298.

Niazi A.K. and R. Sadaf. 2014. Measles Epidemic in Pakistan: In Search of Solutions. *Ann. Med. Health Sci. Res.* 4(1): 1–2.

Nossal G.J. 2000. Inactivated measles vaccine and the risk of adverse events. *Bulletin WHO* 78: 224–225.

Ogundiji O.T., I.O. Okono and F.D. Adu. 2013. Determination of measles hemagglutination inhibiting antibody levels among children in Ibadan, Nigeria. *J. Immunoass. Immunochem.* 34(2): 208–217.

Perry R.T. and N.A. Halsey. 2004. The clinical significance of measles. *J. Infect. Dis.* 189(1): 456–461.

Rasool M.H., M. Saqalein, T. Saeed, M.A. Zahoor, M.I. Najeeb and A.B. Siddique. 2016. Sero-epidemiology of measles in children from district Faisalabad, Pakistan. *Pak. J. Sci.* 68(2): 115–120.

Ratnam S., R. Hamkar, T. Mohktari-Azad, M. Gray, G. Parkyn, C. Head and A.G. Tipples. 2003. Assessment of immunoglobulin M enzyme immunoassays for diagnosis of measles. *J. Clin. Microbiol.* 41: 4790–4792.

Ruzauskas M., M. Virgailis and V. Špakauskas. 2005. Serological diversity and antimicrobial resistance of Salmonella isolated from different sources in Lithuania. *Veterinarski Arhiv.* 75(3): 211–221.

Sakata H. and A. Sugiura. 1988. Passive hemagglutination test for measles immunity and serodiagnosis. *J. Clin. Microbiol.* 26(4): 636.

Shakurnia A., S.M. Alavi, R. Norouzirad, A. Arsalan and G. Shakerinejad. 2013. Post-vaccination immunity against measles in under twenty-five-year-old population of Ahvaz, Southwest of Iran. *Jundish. J. Microbiol.* 6(10): 7707.

Smaron M.F., M. Grandien, E. Osterhaus, P.A. Rota and T.F. Wild. 1991. Laboratory diagnosis of measles infection and monitoring of measles immunization: memorandum from a WHO meeting. *Bulletin WHO.* 72: 207–211.

Soltis R.D., M. Diane, J. Morris and I.D. Wilson. 1979. The effect of heat inactivation of serum on aggregation of immunoglobulins. *Immunol.* 36–37.

World Health Organization. 2008. WHO/UNICEF joint statement of global plan for reducing measles mortality, 2006–2010.

Zahoor M.A., M.H. Rasool, M. Waseem, B. Aslam, M.K. Zahoor, M. Saqalein, Z. Nawaz and S. Sahar. 2015. Prevalence of measles in vaccinated and non-vaccinated children. *EXCLI J.* 14: 504–507.

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SHORT COMMUNICATION

Seroprevalence of Selected Zoonotic Agents among Hunters from Eastern Poland

MAŁGORZATA TOKARSKA-RODAK[1], MARCIN WEINER[1], MONIKA SZYMAŃSKA-CZERWIŃSKA[2], ANNA PAŃCZUK[3], KRZYSZTOF NIEMCZUK[2], JACEK SROKA[4], MIROSŁAW RÓŻYCKI[5] and WOJCIECH IWANIAK[6]

[1] Institute of Health Science, Department of Health, Pope John Paul II State School of Higher Education in Biala Podlaska, Poland
[2] Department of Cattle and Sheep Diseases National Veterinary Research Institute in Pulawy, Poland
[3] Institute of Physical Education and Physiotherapy, Pope John Paul II State School of Higher Education in Biala Podlaska, Poland
[4] Department of Parasitology and Invasive Diseases, National Veterinary Research Institute in Pulawy, Poland
[5] Department of Hygiene of Food of Animal Origin, National Veterinary Research Institute, Poland
[6] Department of Microbiology, National Veterinary Research Institute, Poland

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Abstract

The aim of our study was the collection of seroprevalence data for *Toxoplasma gondii*, *Coxiella burnetii*, *Trichinella* spp., and *Francisella tularensis* from hunters in Lublin Province. The antibodies against *T. gondii* and *C. burnetii* were recorded in 38.5% and 16.2% of the sera, respectively. 4.05% of the sera were seropositive for both *T. gondii* and *C. burnetii*. None of the sera tested reacted positively with *F. tulariensis* or *Trichinella* spp. Seroprevalence of *T. gondii* and *C. burnetii* is common among the hunters from Lublin Province. It seems reasonable to undertake similar research among hunters from other regions of eastern Poland.

Key words: *Coxiella burnetii*, *Francisella tularensis*, *Toxoplasma gondii*, *Trichinella* spp., hunters

Zoonotic agents are distributed widely throughout the world and are noted both in livestock and wild animals (Ciszewski *et al.*, 2014; Richard and Oppliger, 2015; EFSA, 2016; Eliášová *et al.*, 2017). The high risk of zoonotic transmission often occurs in individuals with occupational exposure to animals, such as veterinarians, farmers, and hunters. Human infection is usually acquired through direct contact with infected animals, inhalation of contaminated aerosols or close contact with contaminated environment through secretions and excretions from infected animals. Moreover, the transmission of zoonoses is possible via alimentary route after the consumption of raw or undercooked meat as well as drinking raw milk or water. Hunters are extremely exposed to direct contact with wild-living animals as well as dead animals, contaminated water, soil, and tick bites, which are vectors of many pathogens. Therefore, the risk of zoonotic infections is increased in this group (Richard and Oppliger, 2015; Tokarska-Rodak *et al.*, 2016). In 2016, the European Food Safety Authority and the European Centre for Disease Prevention and Control published a report on zoonoses, zoonotic agents, and food-borne outbreaks noted in 2015 in 32 European countries. Among the important zoonotic factors that may pose a threat to public health are: *Toxoplasma gondii*, *Coxiella burnetii*, *Francisella tularensis* and *Trichinella spiralis* (EFSA, 2016). Generally, the prevalence of zoonotic diseases in humans is underestimated in Europe, including Poland. There are only a few reports in available databases about the prevalence of zoonotic agents in hunters. The data about zoonotic diseases in humans are underestimated mainly due to nonspecific symptoms and low awareness of physicians. Therefore, the aim of our study was to collect the seroprevalence data for *T. gondii*, *C. burnetii*, *Trichinella* sp., *F. tularensis* from hunters in eastern Poland.

The blood samples from hunters belonging to the hunting associations from Lublin Province were taken from October 2014 to April 2015 by venipuncture. The Bioethics Committee at the Medical University of Lublin approved the sampling and laboratory testing of the specimens obtained, decision No. KE-0254/177/2014.

* Corresponding author: M. Tokarska-Rodak, Institute of Health Science, Department of Health, Pope John Paul II State School of Higher Education in Biala Podlaska, Poland; **e-mail: rodak.malgorzata@gmail.com**

A total of 148 hunters aged 23–80 (average 53, SD 11.13) including 123 men and 25 women were examined. Sera were separated by centrifugation (10 min at 1400 × g) and stored at –20°C until analysis.

The serum samples were examined for the presence of IgG antibodies against *T. gondii* with the direct agglutination test (DAT), using a commercial kit (Toxo--Screen DA, bioMérieux, France). The test was performed according to the manufacturer's instruction.

Antibodies against of *C. burnetii* antigens (specific for phase I and II) were analyzed by the complement fixation test (CFT; GmbH, Germany and Biomed, France). The dilution of the samples ranged from 1:5 to 1:80. Partial inhibition of hemolysis in a 1:10 dilution was regarded as a positive result.

For the detection of anti-*F. tularensis* antibodies, the serum agglutination test (SAT) was performed. A commercial antigenic preparation Francisella Tularensis Antigen (Becton Dickinson) was used. The test was carried out with the microagglutination method, following the manufacturer's instruction. For each tested serum, two-fold dilutions between 1/10 and 1/80 were tested. The controls (commercial *F. tularensis* antigen, control antigen and negative serum), were used in each plate. The reaction was interpreted as positive at dilutions of 1:40 and higher.

The serum samples taken from the hunters were preserved in the National Reference Laboratory for Trichinellosis in the National Veterinary Research Institute in Pulawy (NRL) and examined for the presence of anti-*Trichinella* antibodies according to accredited protocol in the Instituto Superiore di Sanita (Rome, Italy), as a part of the services that EURLP (European Union Reference Laboratory for Parasites) provides to NRLs (LAB N° 0689 MI-03 Rev. 3 2014 accredited by the Accredia). Microtiter plates coated with *T. spiralis* excretory/secretory (E/S) antigens were used (Gómez--Morales *et al.*, 2008). OD value was measured at 450 nm. Test results were provided in report No. 56/2015.

The data obtained were analyzed statistically using Statistica v.10 software. Chi-square test was performed for nominal features to detect statistically significant dependence. The assumptive level of significance was $p = 0.05$.

Serological screening revealed that 54.7% (81/148) of the samples tested were seropositive, 16.9% (25/148) were classified as doubtful and 35.8% (53/148) were negative. The antibodies were recorded against *T. gondii* and *C. burnetii* in 38.5% and 16.2% of the sera investigated, respectively. None of the tested sera reacted positively to *F. tulariensis* and *Trichinella* spp. The anti-*T. gondii* IgG antibodies were recorded in 60% (15/25) women and 34.1% (42/123) men ($p = 0.042$, χ^2 Pearsons 6.3). The anti-*C. burnetti* antibodies were recorded in 12% (3/25) women and 17.1% (21/123) men ($p = 0.769$, χ^2 Pearson 0.52) (Table I). Interestingly, antibodies against *C. burnetii* and *T. gondii* were found simultaneously in 7.4% (11/148) of the tested sera. Moreover, 4.05% (6/148) the sera were seropositive for *T. gondii* and *C. burnetii* (Table II).

Table I
Results of serological tests for all four microorganisms.

Sex	Serological results			Result of statistical analysis
	Number of sera (%)			
	Seronegative	Seronegative	Doubtful	
T. gondii				
M	76 (61.8)	42 (34.1)	5 (4.1)	$p = 0.042$ $\chi^2 = 6.31$
F	10 (40)	15 (60.0)	0 (0)	
Total	86 (58.1)	57 (38.5)	5 (3.4)	
C. burnetii				
M	85 (69.1)	21 (17.1)	17 (13.8)	$p = 0.769$ $\chi^2 = 0.52$
F	19 (76.0)	3 (12.0)	3 (12.0)	
Total	104 (70.3)	24 (16.2)	20 (13.5)	
F. tularensis				
M	123 (83.1)	–	–	–
F	25 (16.9)	–	–	
Total	148 (100)	–	–	
Trichinella sp.				
M	123 (83.1)	–	–	–
F	25 (16.9)	–	–	
Total	148 (100)	–	–	

Table II
Results of serological tests for *T. gondii* and *C. burnetii*.

T. gondii	*C. burnetii*		
	Number of sera (%)		
	Seronegative	Seropositive	Doubtful
Seronegative sera	53 (35.8)	17 (11.4)	16 (10.8)
Seropositive sera	47 (31.7)	6 (4.05)	4 (2.7)
Doubtful sera	4 (2.7)	1 (0.67)	0 (0)
Total	104 (70.3)	24 (16.2)	20 (13.5)

There was no statistically significant relationship between the age of the subjects and the presence of antibodies among the pathogens tested.

Hunters are one of the groups occupationally exposed to infection with tick borne disease and other zoonotic agents. Hunters may be exposed to contact with live or dead animals as well as their excretions and secretions. Moreover, they are exposed to tick bites, so the transmission of zoonotic agents and tick-borne diseases, *e.g.*, Q fever caused by *C. burnetii* cannot be ruled out. The most common zoonotic disease is toxoplasmosis. It is estimated that approximately 25% – 30% of the global population is infected with *T. gondii*; however, the incidence varies between countries and regions or between different communities within a region. Seroprevalence at the level of 10–30% was noted in North America and northern Europe, while in the countries of central and southern Europe (Gangneux and Dardé, 2012) the percentage ranged from 30 to 50%. The previous reports from Poland revealed that seroprevalence of *T. gondii* were close to the world's average (Kapka *et al.*, 2010; Sroka *et al.*, 2010; Milewska-Bobula *et al.*, 2015). Our investigation showed a very similar level of seroprevalence for *T. gondii* among hunters (41.9%). Parallelly, in 37.5% of the sera antibodies against *C. burnetti* were detected. The previous reports showed anti-*T. gondii* IgG antibodies in employees of forest inspectorates and their family members in eastern Poland (61.4%) (Sroka and Szymańska, 2012), farmers (66.9%) (Sroka *et al.*, 2010), and meat processing industry workers (65.4%) (Sroka *et al.,* 2003). People living in rural households are infected with *T. gondii* more frequently (66.9%) than city dwellers (41%), and the incidence of the infection increases with age (Sroka *et al.,* 2010). Szymańska-Czerwińska *et al.* (2015) estimated seropositivity for *C. burnetti* among humans occupationally exposed to zoonoses at 31.12%, 39.07%, and 15.23% in IFA, ELISA, and CF methods, respectively.

Q fever outbreaks caused by *C. burnetii* are very common in the world, but in many countries human data are very limited. In the report from 2015, 833 confirmed cases of Q fever were reported in the EU (the notification rate was 0.16 per 100 000 of the population). The highest notification rate was observed in Spain (0.54), Croatia (0.49), Cyprus (0.47), France and Germany (both 0.38), and Hungary (0.35), while Estonia, Iceland, Lithuania, Malta, and Slovakia reported no human cases in 2015 (EFSA, 2016). In Poland, one case of Q fever was reported in 2014 (incidence 0.003/100 000), and no cases were reported in 2015 (Czarkowski *et al.,* 2016). Taking into consideration that Q fever outbreaks have been noted in cattle and small ruminants in recent years, it is very probable that these data are underestimated. Our results are comparable to that reported by Szymańska-Czerwińska *et al.* (2015).

Another subject of our survey was tularemia. In European countries, the highest prevalence of tularemia in 2001–2010 was noted in Kosovo (incidence 5.2/100 000), Sweden (incidence 2.80/100 000), and Finland (incidence 1.19/100 000) (Gürcan, 2014). As reported by the National Institute of Public Health – National Institute of Hygiene, in Poland there were 11 tularemia cases (incidence 0.029/100 000) in 2014 and 9 cases (incidence 0.023/100 000) in 2015 (Czarkowski *et al.,* 2016). No anti-*F. tularensis* antibodies were detected in the serum samples from hunters in this study. The number of tularemia cases in Poland may be underrated due to the widespread use of aminoglycoside antibiotics and fluoroquinolones as second-line drugs used in adults to treat soft tissue and lymph node infections, which eliminate the tularemia symptoms without diagnosing the disease (Weiner and Kubajka, 2015). In the field of food safety, nematode parasites of the genus *Trichinella* still represent a concern for the public health due to hundreds of human infections documented yearly as the outcome of the consumption of wild boar meat (Murrell and Pozio, 2011; EFSA, 2012). *T. spiralis* foci have been present in Poland in domestic and wild animals, but other *Trichinella* species, such as *Trichinella britovi*, *Trichinella pseudospiralis* and *Trichinella nativa* have been detected in wildlife in the last decade (Chmurzyńska *et al.*, 2013; Bilska-Zając *et al.*, 2016; Bilska-Zając *et al.*, 2017). The consumption of unexamined pork causes average incidence of 0.9 cases per million persons per year (Mpy). The incidence caused by the consumption of wild boar meat is twice as high

and is estimated at 1.97 Mpy. Since wild boar meat is the most important source for trichinellosis outbreaks in humans, this study was aimed at evaluating the seroprevalence within the group at high risk. Hunters are recognized as the group of high risk due to their hobby.

Seroprevalence of *T. gondii* and *C. burnetii* is common among the hunters from Lubelskie Province while antibodies against *F. tularensis* and *T. spiralis* are absent. It seems reasonable to undertake similar research among hunters from other regions of eastern Poland. Knowledge in this field might be of importance for public health.

Literature

Bilska-Zając E., M. Różycki, E. Chmurzyńska, E. Antolak, M. Próchniak, K. Grądziel-Krukowska, J. Karamon, J. Sroka, J. Zdybel and T. Cencek. 2017. First case of *Trichinella nativa* infection in wild boar in Central Europe-molecular characterization of the parasite. *Parasitol. Res.* 116(6): 1705–1711.

Bilska-Zając E., M. Różycki, J. Karamon, J. Sroka, M. Próchniak, E. Antolak, E. Chmurzyńska and T. Cencik. 2016. First record of wild boar infected with *Trichinella pseudospiralis* in Poland. *Bull. Vet. Inst. Pulawy.* 60(2): 147–152.

Chmurzynska E., M. Rozycki, E. Bilska-Zajac, K. Nockler, A. Mayer-Scholl, E. Pozio, T. Cencek and J. Karamon. 2013. *Trichinella nativa* in red foxes (*Vulpes vulpes*) of Germany and Poland: possible different origins. *Vet. Parasitol.* 198: 254–257.

Ciszewski M., T. Czekaj and E.M. Szewczyk. 2014. New insight into bacterial zoonotic pathogens posing health hazards to humans. *Med. Pr.* 65(6): 819–829.

Czarkowski M.P., E. Cielebąk, E. Staszewska-Jakubik and B. Kondej. 2016. National Institute of Public Health – National Institute of Hygiene – Department of Epidemiology, Chief Sanitary Inspectorate – Department for Communicable Disease and Infection Prevention and Control, Infectious diseases and poisonings in Poland in 2015. http://wwwold.pzh.gov.pl/oldpage/epimeld/2015/Ch_2015.pdf, 05.10.2017.

EFSA (European Food Safety Authority). 2012. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2010. *EFSA Journal.* 10: 2597–3039.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). 2016. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA Journal* 14(12): 4634.

Eliášová A., M. Tokarska-Rodak, K. Laskowski, E. Pawłowicz, M. Fiedoruk, D. Magurová and W. Mikuľáková. 2017. Knowledge of nursing students about the transmission and prevention of infections caused by *Toxoplasma gondii*, Rubella virus, Cytomegalovirus in women during pregnancy. *Health Problems of Civilization.* 11(1): 40–44.

Gangneux F.R. and M.L. Dardé. 2012. Epidemiology of and diagnostic strategies for toxoplasmosis. *Clin. Microbiol. Rev.* 25(2): 264–296.

Gómez-Morales M.A., A. Ludovisi, M. Amati, S. Cherchi, P. Pezzotti and E. Pozio. 2008. Validation of an enzyme-linked immunosorbent assay for diagnosis of human trichinellosis. *Clin. Vaccine. Immunol.* 15(11): 1723–1729.

Gürcan Ş. 2014. Epidemiology of Tularemia. *Balkan. Med. J.* 31: 3–10.

Kapka L., K. Perżyło, M. Cyranka, M. Skrzypczak and L. Wdowiak. 2010. Congenital toxoplasmosis as a relevant health problem. *Zdr Publ* 120(1): 80–86.

Milewska-Bobula B., B. Lipka, E. Gołąb, R. Dębski, M. Marczyńska, M. Paul, A. Panasiuk, M. Seroczyńska, J. Mazela and D. Dunin-Wąsowicz. 2015. Recommended management of *Toxoplasma gondii* infection in pregnant women and their children. *Przegl. Epidemiol.* 69: 291–298.

Murrell K.D. and E. Pozio. 2011. Worldwide occurrence and impact of human trichinellosis, *Emerg. Infect. Dis.* 17: 2194–2202.

Richard S. and A. Oppliger. 2015. Zoonotic occupational diseases in forestry workers – Lyme borreliosis, tularemia and leptospirosis in Europe. *Ann. Agr. Env. Med.* 22(1): 43–50.

Sroka J. and J. Szymańska. 2012. Analysis of prevalence of *Toxoplasma gondii* infection in selected rural households in the Lublin region. *Bull. Vet. Inst. Pulawy.* 56, 529–534.

Sroka J., A. Wójcik-Fatla, J. Szymańska, J. Dutkiewicz, V. Zając and J. Zwoliński. 2010. The occurrence of *Toxoplasma gondii* infection in people and animals from rural environment of Lublin region – estimate of potential role of water as a source of infection. *Ann. Agric. Environ. Med.* 17: 111–118.

Sroka J., J. Zwoliński and J. Dutkiewicz. 2003. The prevalence of *Toxoplasma gondii* antibodies among abattoir workers in Lublin. *Wiad. Parazytol.* 49: 47–55.

Szymańska-Czerwińska M., E.M. Galińska, K. Niemczuk and J.P. Knap. 2015. Prevalence of *Coxiella burnetii* infection in humans occupationally exposed to animals in Poland. *Vector. Borne. Zoonotic. Dis.* 15: 261–267.

Tokarska-Rodak M., D. Plewik, A.J. Michalski, M. Kołodziej, A. Mełgieś, A. Pańczuk, H. Konon and M. Niemcewicz. 2016. Serological surveillance of vector-borne and zoonotic diseases among hunters in eastern Poland. *J. Vector. Borne. Dis.* 53: 355–361.

Weiner M. and M. Kubajka 2015. Tularemia – serious zoonotic disease. *Health Problems of Civilization* 9: 39–46.

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SHORT COMMUNICATION

New Gene Responsible for Resistance of Clinical Corynebacteria to Macrolide, Lincosamide and Streptogramin B

MAGDALENA SZEMRAJ[1]*, ANNA KWASZEWSKA[2] and ELIGIA M. SZEWCZYK[1]

[1] Department of Pharmaceutical Microbiology and Microbiological Diagnostics, Medical University of Łódź, Łódź, Poland
[2] Institute of Health Sciences, State College of Applied Sciences in Skierniewice, Skierniewice, Poland

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Abstract

The subject of the study was phenotypic marking of the antibiotic susceptibility and MLS_B resistance mechanism in *Corynebacterium* spp. isolated from human skin (18 isolates) and from clinical materials (19 isolates). The strains were tested for the presence of the *erm*(A), *erm*(B), *erm*(C), *erm*(X), *lnu*(A), *msr*(A), *msr*(B) and *mph*(C) genes. Clinical isolates showed wide resistance to antibiotics. In 89% clinical isolates and 72% skin microbiota a constitutive type of MLS_B resistance was found. In 12 clinical isolates the *erm*(C) gene was detected-eight of which had *erm*(X) as well as *erm*(C), two harboured *erm*(X), *erm*(C) and *erm*(A) and two demonstrated only *erm*(C).

Key words: *Corynebacterium* spp., *erm*(C), MLS_B, resistance genes

Escalating resistance of bacteria to antibiotics, including macrolides, leads to progressive difficulties in treatment of bacterial infections. The problems also concern infections caused by opportunistic corynebacteria (Ortiz-Perez *et al*., 2010; Olender, 2013; Alibi *et al*., 2007). Resistance to macrolides often results from the enzymatic methylation of adenosine of the 23S rRNA ribosomal subunit and is encoded by the genes from the *erm* family. The above-described mechanism affects resistance to lincosamides and streptogramin B and so that it is referred to as the MLS_B (Maravic, 2004). Phenotypic expression of MLS_B resistance can be constitutive or inducible. Strains with constitutive MLS_B ($cMLSB_B$) resistance are considered resistant to all macrolides, lincosamides and streptogramin B, whereas strains with inducible MLS_B ($iMLS_B$) present resistance to 14- and 15-member macrolides but appear susceptible to 16-member macrolides, lincosamides and type B streptogramins (Weisblum, 1995). Detection of any of these mechanisms eliminates such groups of antibiotics from the therapy. Additionally, resistance to macrolides, lincosamides and streptogramin B may also be caused by other mechanisms such as efflux pumps or enzymatic inactivation of antibiotics encoded by other groups of genes (Roberts *et al*., 1999; Roberts, 2008).

Resistance to marcolides, lincosamides, and streptogramin B in *Corynebacterium* spp. is noticeable especially in clinical multidrug-resistant isolates (Otsuka *et al*., 2006; Ortiz-Perez *et al*., 2010). Nevertheless, in a manuscript published in Microbial Drug Resistance in 2014 (Szemraj *et al*., 2014), we have shown that this resistance may also occur in the skin microbiota *e.g. C. tuberculostearicum* or *C. jeikeium*. Our study covered 99 strains and confirmed earlier reports that the gene responsible for this mechanism in corynebacteria is *erm*(X). During that study we did not find other genes whereas other researchers demonstrated existence of the *erm*(B), *mef* or *msr*(A) genes in these bacteria (Luna *et al*., 1999; Ojo *et al*., 2006; Ortiz-Perez *et al*., 2010).

In the following years we pursued the studies on new groups of strains. The aim of the research was phenotypic marking of the antibiotic susceptibility and detecting of MLS_B resistance mechanism in *Corynebacterium* spp. For the investigation we used 18 strains from skin of healthy young men that did not have long-term contact with the healthcare services (natural microbiota) and 19 strains from clinical materials (mainly from blood, urine and wounds).

The strains were identified by the MALDI-TOF/VITEK MS mass spectrometry system (bioMerieux), performed in the Synevo laboratory in Łódź, and by the Coryne API (bioMerieux) phenotypic method. Among the clinical isolates and skin microbiota tested there were lipophilic as well as non-lipophilic species. Sensitivity to penicillin, tetracycline, erythromycin, clindamycin, gentamycin, ciprofloxacin, trimethoprim/sulfamethoxazole,

* Corresponding author: M. Szemraj, Department of Pharmaceutical Microbiology and Microbiological Diagnostics, Medical University of Łódź, Łódź, Poland; **e-mail: magdalena.szemraj@umed.lodz.pl**

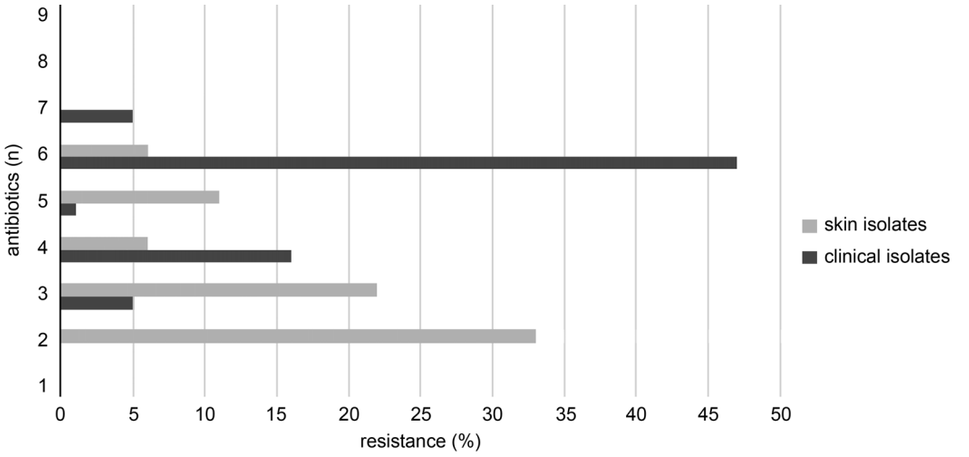

Fig. 1. Percentage of strains resistant to two or more antibiotics among skin microbiota and clinical isolates.

tigecycline and linezolid was being marked by the disc diffusion method according to the EUCAST guidelines. *S. pneumoniae* ATCC 49619 was used as a control strain. In the breakpoints absence, CLSI guidelines for *Corynebacterium* spp. were used. When it was not possible to apply such criteria, EUCAST guidelines were administered as for *Staphylococcus* spp. Both the inducible and constitutive resistance mechanisms were phenotypically determined in compliance with the EUCAST guidelines for *Staphylococcus* species by the application of *S. aureus* ATCC 29213 as the control strain. DNA was isolated with the usage of Genomic Mini (A&A Biotechnology) commercial set in line with the manufacturer's protocol. PCR was performed by the application of the primers (Genomed, Sequencing Laboratory, Poland) specific for the *erm*(A), *erm*(B), *erm*(C) (Sutcliffe *et al.*, 1996), *erm*(X) (Rosato *et al.*, 2001), *mph*(C) (Perreten *et al.*, 2005), *msr*(A) (Ojo *et al.*, 2006), *msr*(B) and *lnu*(A) (Lina *et al.*, 1999) genes. DNA amplification was carried out in the thermal cycler (Biometra). PCR were initialized by a denaturation step (5 min at 94°C), followed by 30 cycles of amplification (denaturation 30 sec at 94°C, annealing 30 sec at 46–56°C, DNA chain extension 1 min at 72°C), and a final extension step (10 min at 72°C). Reaction products were recognized by electrophoresis (70V by 1 h) in 1% (w/v) agarose gels with Midori Green DNA Stain (NIPPON Genetics EUROPE). For the readout of outcomes the CCD camera (Syngen) was used. Due to a commercial molecular size marker the amplification products sizes were determined using DraMix (A&A BIOTECHNOLOGY). *C. jeikeium* K411 (CCUG 27385) harbouring *erm*(X) and *S. epidermidis* strains: 3718INL harbouring *erm*(B) and *mph*(C), 1486IG with *erm*(A), *erm*(C) and *mph*(C) and 1923KIINL with the *msr*(A) and *lnu*(A) genes were used as positive controls (Juda, 2010).

Similarly to the former work, the identification of strains occurred to be tough. However, by the usage of MALDI-TOF mass spectrometry (that was based on the analysis of the protein profiles of the tested strains) and phenotypic methods we managed to identify most of the tested strains i.e. 17 clinical isolates and 13 isolates of skin microbiota. None of genetic methods was applied. For the *Corynebacterium* spp., the sequencing of the 16S rRNA subunit gene limits the low polymorphism of such sequence between species. Thus, sequencing the *rpoB* gene is the only possibility (Khamis *et al.*, 2004). Among clinical isolates *C. striatum* and *C. jeikeium* prevailed, while among the skin microbiota *C. tuberculostearicum* and *C. jeikeium* predominated. *C. jeikeium*, similarly to other corynebacteria, is often described as the cause of infection and less often as a sample contamination (Wang *et al.*, 2001; Akan *et al.*, 2002; Mookadam *et al.*, 2006). *C. jeikeium* and *C. striatum* were isolated from endocarditis, bones, joints, lungs, cerebrospinal and bacteremia (Lee *et al.*, 2005; Tleyjeh *et al.*, 2005; Belmares *et al.*, 2007; Chen *et al.*, 2012; Daisuke *et al.*, 2017).

The clinical isolates (especially C. *striatum*, *C. amycolatum* and *C. jeikeium*) showed a wide resistance to antibiotics. All strains from this group were resistant to at least three antibiotics (Fig. 1), nine strains to six antibiotics and five to seven antibiotics (three strains of *C. striatum*, one of *C. amycolatum*/*xerosis* and one of *C. glucuronolyticum*).

Soriano *et al.* (1995), Reddy *et al.* (2012) and Hahn *et al.* (2016) also pointed out the growing resistance of corynebacteria isolated from clinical materials. Among the skin microbiota five strains were sensitive to all tested antibiotics whereas the others were resistant to at least two up to six antibiotics. All strains from both groups were sensitive to linezolid and tigecycline. Similar *Corynebacterium* spp. sensitivity to these antibiotics was also presented by Morata *et al.* (2014), Tang *et al.* (2015) and Alibi *et al.* (2007). The constitutive type of MLS_B resistance was demonstrated in 17 strains tested from clinical specimens (89%) while the other two iso-

Table I
Distribution of resistance genes among clinical isolates and skin microbiota.

Resistance phenotype	Resistance genes (number of isolates and species)			
	erm(X)	*erm*(C)	*erm*(X)+*erm*(C)	*erm*(X)+*erm*(C)+*erm*(A)
Clinical isolates	*C. striatum* *C. glucuronolyticum* *C. amycolatum/xerosis*	*C. aurimucosum*	*C. jeikeium* *C. striatum* *C. aurimucosum*	*C. pseudituberculosis* *Corynebacterium* sp.
E^RC^R	6	1	8	2
E^RC^S	0	1	0	0
E^SC^R	0	0	1	0
Skin microbiota	*C. jeikeium* *C. tuberculostearicum* *Corynebacterium* sp.			
E^RC^R	13	0	0	0

E^RC^R – resistant both to erythromycin and clindamycin, E^RC^S – resistant to erythromycin and susceptible to clindamycin, E^SC^R –susceptible to erythromycin and resistant to clindamycin

lates were resistant to one from two antibiotics. This resistance was accompanied by the *erm*(A), *erm*(C) and *erm*(X) genes – individually or in groups. The *erm*(B), *msr*(A), *msr*(B), *mph*(C) and *lnu*(A) genes were not found in the genomes of any of the strains tested. The constitutive MLS_B resistance mechanism was also demonstrated in 13 resistant strains isolated from healthy human skin (72%). These strains were harboring only the *erm*(X) gene what has confirmed our previous observations (Szemraj *et al.*, 2014).

It draws the attention that we detected for the first time a large number of clinical isolates of *Corynebacterium* spp. which alongside with the commonly described genes such as *erm*(X) (Olender and Niemcewicz, 2010; Olender, 2013) were harbouring also *erm*(C). In two isolates, three genes: *erm*(X), *erm*(C) and *erm*(A) simultaneously existed. In other two isolates (the first resistant to macrolide and lincosamides and the second only to macrolide), solely *erm*(C) was detected (Table I).

These results indicate the importance of this gene in the spreading of MLS_B resistance mechanism in *Corynebacterium* spp. which until now has not been frequently sought in corynebacteria in spite of the fact that it is often found in staphylococci (Chaieb *et al.*, 2007; Cetin *et al.*, 2010).

The results obtained show that clinical strains derived from the strains accounting for natural microbiota easily acquire new antibiotic resistance genes. The fact mentioned above was proven by the resistance profile of the strains tested including genes that provide resistance to MLS_B antibiotics. There is a possibility of horizontal gene transfer in these bacteria what can be suggested by the different location of the *erm*(X) gene in the *Corynebacterium* spp. In *C. diphteriae* it is a part of 14.5 kpz the plasmid pNG2, in *C. xerosis* this gene is a part of the Tn5432 transposon located on the 50 kbp Ptp10 plasmid, whereas in *C. jeikeium* and *C. striatum* it is often located on the Tn5432 transposon inside the chromosome (Roberts *et al.*, 1992; Tauch *et al.*, 1995, 2003, 2005; Rosato *et al.*, 2001). Tn5432 transposon harboring *erm*(X) is also found in other *Propionibacterium* spp. (Ross *et al.*, 2002; El-Mahdy *et al.*, 2010). Our finding of the *erm*(C) gene among *Corynebacterium* spp. that is responsible for the MLS_B mechanism mainly in coagulase-negative staphylococci, indicates the potential for gene acquisition by clinical strains from these bacteria (Chaieb *et al.*, 2007; Gatermann *et al.*, 2007; Cetin *et al.*, 2010).

Acknowledgments

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Literature

Akan A., J. Bengisun, A. Bati, M. Arat, O. Ilhan and H. Akan. 2002. A case of catheter related septicemia caused by *Corynebacterium jeikeium*. *Turk. J. Haematol.* 19: 47–50.

Alibi S., A. Ferjani, J. Boukadida, M.E. Cano, M. Fernández-Martínez, L. Martínez-Belmares J., S. Detterline, J.B. Pak and J.P. Parada. 2007. *Corynebacterium* endocarditis species – specific risk factors and outcomes. *BMC Infect. Dis.* 7: 4.

Cetin E.S., H. Gunes, S. Kaya, B.C. Aridogan and M. Demirci. 2010. Distribution of genes encoding resistance to macrolides, lincosamides and streptogramins among clinical staphylococcal isolates in a Turkish University Hospital. *J. Microbiol. Immunol. Infect.* 43: 524–529.

Chaieb K., T. Zmantar, O. Chehab, O. Bouchami, A.B. Hasen and K. Mahdouani. 2007. Antibiotic resistance genes detected by multiplex PCR assays in *Staphylococcus epidermidis* strains isolated from dialysis fluid and needles in a dialysis service. *Jpn. J. Infect. Dis.* 60: 183–187.

Chen F.L., P.R. Hsueh, S.O. Teng, T.Y. Ou, W.S. Lee 2012. *Corynebacterium striatum* bacteremia associated with central venous catheter infection. *J. Microbiol. Immunol. Infect.* 45: 255–258.

CLSI, Clinical and Laboratory Standards Institute. 2015. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline – 3nd ed. M45, Wayne, PA, USA.

Daisuke U., T. Oishi, K. Yamane and K. Terada. 2017. *Corynebacterium striatum* bacteremia associated with a catheter-related blood stream infection. 2017. *Case Rep. Infect. Dis.* 2017: 2682149.

El-Mahdy T.S., S. Abdalla, R. El-Domany, M.S. Mohamed, J.I. Ross and A.M. Snelling. 2010. Detection of a new *erm*(X)-mediated antibiotic resistance in Egyptian cutaneous propionibacteria. *Anaerobe* 16: 376–379.

EUCAST, European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.1, valid from 2017.03.13.

Gatermann S.G., T. Koschinski and S. Friedrich. 2007. Distribution and expression of macrolide resistance genes in coagulase-negative staphylococci. *Clin. Microbiol. Infect.* 13: 777–781.

Hahn W. O., J. B. Werth, S. M. Butler-Wu and R. M. Rakita. 2016. Multidrug-resistant *Corynebacterium striatum* associated with increased use of parenteral antimicrobial drugs. *Emerg. Infect. Dis.* 22: 1908–1914.

Juda M. 2010. PhD. thesis, Phenotypic and genetic analysis of coagulase-negative staphylococci resistance to macrolides, lincosamides and streptogramins (in Polish). Department of Pharmaceutical Microbiology with Laboratory for Microbiological Diagnostics, Medical University of Lublin. Lublin. Poland.

Khamis A., D. Raoult and B. La Scola. 2004. rpoB gene sequencing for identification of *Corynebacterium* species. *J. Clin. Microbiol.* 42: 3925–3931.

Lee P.P., D.A. Ferguson and F.A. Sarubbi. 2005. *Corynebacterium striatum*: an underappreciated community and nosocomial pathogen. *J. Infect.* 50: 338–343.

Lina G., A. Quaglia, M. Reverdy, R. Leclercq, F. Vandenesch, and J. Etienne. 1999. Distribution of genes encoding resistance to macrolides, lincosamides and streptogramins among staphylococci. *Antimicrob. Agents Chemother.* 43: 1062–1066.

Luna V.A., P. Coates, A. Eady, J.H Cove., T.T.H. Nguyen and M.C. Roberts. 1999. A variety of Gram-positive bacteria carry mobile *mef* genes. *J. Antimicrob. Chemother*. 44: 19–25

Maravic G. 2004. Macrolide resistance based on the Ermmediated rRNA methylation. *Curr. Drug Targets Infect. Disord.* 4: 193–202.

Mookadam F., M. Cikes, L.M. Baddour, I.M. Tleyjeh and M. Mookadam. 2006. *Corynebacterium jeikeium* endocarditis: a systematic overview spanning four decades. *Eur. J. Clin. Microbiol. Infect. Dis.* 25: 349–353.

Morata L., E. Tornero, J.C. Martínez-Pastor, S. García-Ramiro, J. Mensa and A. Soriano. 2014. Clinical experience with linezolid for the treatment of orthopaedic implant infections. *J. Antimicrob. Chemother*. 69: 47–52.

Ojo K.K., M.J. Striplin, C.C. Ulep, N.S. Close, J. Zittle, H. Luis, J. Leitao, M.C. Roberts and M. Bernardo. 2006. Staphylococcus efflux *msr*(A) gene characterized in *Streptococcus*, *Enterococcus*, *Corynebacterium*, and *Pseudomonas* isolates. *Antimicrob. Agents Chemother.* 50: 1089–1091.

Olender A. 2013. Antibiotic resistance and detection of the most common mechanism of resistance (MLSB) of opportunistic *Corynebacterium*. *Chemotherapy* 59: 294–306

Olender A. and M. Niemcewicz. 2010. Macrolide, lincosamide, and streptogramin B-constitutive-type resistance in *Corynebacterium pseudodiphtheriticum* isolated from upper respiratory tract specimens. *Microb. Drug Resist.* 16: 119–122.

Ortiz-Perez A., N.Z. Martin-de-Hijas, J. Esteban, M.I. Fernandez-Natal, J.I. Garcia-Cia and R. Fernandez-Roblas. 2010. High frequency of macrolide resistance mechanisms in clinical isolates of *Corynebacterium* species. *Microb. Drug Resist.* 16: 273–277.

Otsuka Y., K. Ohkusu, Y. Kawamura, S. Baba, T. Azeki and S. Kimura. 2006. Emergence of multidrugresistant *Corynebacterium striatum* as a nosocomial pathogen in long-term hospitalized patients with underlying diseases. *Diagn. Microbiol. Infect. Dis.* 54: 109–114.

Perreten V., L. Vorlet-Fawer, P. Slickers, R. Ehricht, P. Kuhnert, and J. Frey. 2005. Microarray-based detection of 90 antibiotic resistance genes of gram-positive bacteria. *J. Clin. Microbiol.* 43: 2291–2302.

Reddy B.S., A. Chaudhury, U. Kalawat, R. Jayaprada, G.S.K. Reddy and B.V. Ramana. 2012. Isolation, speciation and antibiogram of clinically relevant non-diphterial Corynebacteria (Diphtheroids). *Indian J. Med. Microbiol.* 30: 52–57.

Roberts M.C. 2008. Update on macrolide-lincosamide streptogramin, ketolide, and oxazolidinone resistance genes. *FEMS Microbiol. Lett.* 282: 147–159.

Roberts M.C., R.B. Leonard, A. Briselden, F.D. Schoenknecht and M.B. Coyle. 1992. Characterization of antibiotic-resistant *Corynebacterium striatum* strains. *J. Antimicrob. Chemother.* 30: 463–474.

Roberts M.C., J. Sutcliffe, P. Courvalin, L.B. Jensen, J. Rood, and H. Seppala. 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob. Agents Chemother.* 43: 2823–2830.

Rosato A.E., B.S. Lee and K.A. Nash. 2001. Inducible macrolide resistance in *Corynebacterium jeikeium*. *Antimicrob. Agents Chemother.* 45: 1982–1989.

Ross J.I., A.A. Eady, E. Carnegle and J.H. Cove. 2002. Detection of transposon Tn5432-mediated macrolide-lincosamide-streptogramin B (MLSB) resistance in cutaneus propionibacterie from six European cities. *J. Antimicrob. Chemother.* 49: 165–168.

Soriano F., J. Zapardiel and E. Nieto. 1995. Antimicrobial susceptibilities of *Corynebacterium* species and other non-spore-forming gram-positive bacilli to 18 antimicrobial agents. *Antimicrob. Agents Chemother.* 39: 208–214.

Sutcliffe J., T. Grebe, A. Tait-Kamradt, and L. Wondrack. 1996. Detection of erythromycin-resistant determinants by PCR. *Antimicrob. Agents Chemother.* 40: 2562–2566.

Szemraj M, A. Kwaszewska, R. Pawlak and E.M. Szewczyk. 2014. Macrolide, lincosamide, and streptogramin B resistance in lipophilic corynebacteria inhabiting healthy human skin. *Microb. Drug Resist.* 20: 404–409.

Tang S., L. Yao, X. Hao, X. Zhang, G. Liu, X. Liu, M. Wu, L. Zen, H. Sun, Y. Liu and others. 2015. Efficacy, safety and tolerability of linezolid for the treatment of XDR-TB: a study in China. *Eur. Respir. J.* 45: 161–170.

Tauch A., N. Bischoff, I. Brune and J. Kalinowski. 2003. Insights into the genetic organization of the *Corynebacterium diphtheriae* erythromycin resistance plasmid pNG2 deduced from its complete nucleotide sequence. *Plasmid*. 49: 63–74.

Tauch A., O. Kaiser, T. Hain, A. Goesmann, B. Weisshaar, A. Albersmeier, T. Bekel, N. Bischoff, I. Brune, T. Chakraborty and others. 2005. Complete genome sequence and analysis of the multiresistant nosocomial pathogen *Corynebacterium jeikeium* K411, a lipidrequiring bacterium of the human skin flora. *J. Bacteriol.* 187: 4671–4682.

Tauch A., F. Kassing, J. Kalinowski and A. Pühler. 1995. The *Corynebacterium xerosis* composite transposon Tn5432 consists of two identical insertion sequences, designated IS1249, flanking the erythromycin resistance gene *erm*CX. *Plasmid* 34: 119–131.

Tleyjeh I.M., M.O. Qutub, M. Bakleh, M.R. Sohail and A. Virk. 2005. Corynebacterium jeikeium prosthetic joint infection: case report and literature review. *Scand. J. Infect. Dis.* 37: 151–153.

Wang C., D. Mattson and A. Wald. 2001. *Corynebacterium jeikeium* bacteriemia in bone marrow transplant patients with Hickman catheters. *Bone Marrow Transplant* 27: 445–449.

Weisblum B. 1995. Erythromycin resistance by ribosome modification. *Antimicrob. Agents Chemother.* 39: 577–585.

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SHORT COMMUNICATION

Development and Evaluation of a Latex Agglutination Test for the Identification of *Francisella tularensis* Subspecies Pathogenic for Human

WALDEMAR RASTAWICKI[1], KAMILA FORMIŃSKA[2] and ALEKSANDRA A. ZASADA[2*]

[1] Department of Bacteriology, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland
[2] Department of Sera and Vaccines Evaluation, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland

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Abstract

Francisella tularensis are highly infectious bacteria causing a zoonotic disease called tularemia. Identification of this bacterium is based on antigen detection or PCR. The paper presents a latex agglutination test (LAT) for rapid identification of clinically relevant *F. tularensis* subspecies. The test can be performed within three minutes with live or inactivated bacteria. The possibility to test the inactivated samples reduces the risk of laboratory acquired infection and allows performing the test under BSL-2 conditions.

Key words: *Francisella tularensis*, *F. tularensis* identification, latex agglutination test, tularemia, zoonosis

Tularemia is a zoonotic, highly infectious disease caused by an intracellular Gram-negative bacterium, *Francisella tularensis*. The disease affects a wide range of hosts including invertebrates, mammals and birds. Humans can become infected by direct contact with an infected animal (through broken skin, scratch or tissue injury), through a bite of haematophagous arthropods (*e.g.* fleas, lice, midges, bedbugs, mosquitoes, ticks), by drinking contaminated water, eating contaminated food, or through inhalation of contaminated dust (Formińska *et al.*, 2015). The clinical presentation in humans depends on the route of infection and varies in symptoms and severity (Eliasson *et al.,* 2006). There are four subspecies of *F. tularensis: tularensis*, *novicida*, *mediasiatica* and *holarctica.* Of these, subsp. *tularensis* and subsp. *holarctica* cause disease in humans, whereas subsp. *mediasiatica* is believed to be of relatively low virulence in humans, and only rare cases of human disease caused by this subspecies are known. *F. tularensis* subsp. *novicida* is non-pathogenic for humans (Celli and Zahrt, 2013).

According to WHO case definition, a confirmation of tularemia case requires recovery of an isolate and identification of the culture as *F. tularensis* by antigen or DNA detection. Commercial biochemical identification systems available in clinical diagnostic laboratories are not suitable for accurate identification of *F. tularensis*. Alternatively, paired serum specimens with a fourfold difference in titre (tube or microagglutination assay) or significantly (ELISA), with at least one serum positive, are also considered confirmatory (WHO, 2007). However, antibody against *F. tularensis* are detectable in patients' serum 10–20 days post-infection (Koskela and Salminen, 1985). Thus, usefulness of antibody detection tests is limited in severe cases and when a rapid preventive action must be undertaken. On the other hand, a *F. tularensis* antiserum (Becton Dickinson Diagnostic Systems) recommended by WHO for the slide agglutination test for *F. tularensis* culture identification has been withdrawn from the manufacturer's offer and is not available on the market any more. Below we described the latex agglutination test (LAT) for the rapid identification of *F. tularensis* isolates that could be an alternative for the classical slide agglutination test.

For the preparation of sera for coating of latex beads we used pooled serum samples obtained from 25 patients with high level of IgM antibodies to *F. tularensis,* specified as OD_{450} higher than 1,80 by ELISA (Rastawicki and Wolaniuk, 2013; Rastawicki *et al.,* 2015). The gamma globulin fractions of sera were isolated by 40% ammonium sulfate fractionation in cold-bath, and the precipitate was resuspended in phosphate-buffered saline (PBS), pH 7.4. The solution was subsequently dialyzed against PBS for 48 hours until

* Corresponding author: A.A. Zasada, Department of Sera and Vaccines Evaluation, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland; **e-mail: azasada@pzh.gov.pl**

Table I
Bacterial strains used in the study and results of the latex agglutination test (LAT) after 1, 3 and 5 minutes of rocking.

Microorganism	Result of latex agglutination test*		
	1 min	3 min	5 min
Francisella tularensis ssp. *holarctica* A 104-15	+	+++	+++
Francisella tularensis ssp. *holarctica* LVS	+	+++	+++
Francisella tularensis ssp. *tularensis* Ft33	+	+++	+++
Francisella tularensis ssp. *novicida* Ft26	–	–	–
Yersinia enterocolitica ATCC 23715	–	–	–
Shigella sonnei WHO SH 16-1	–	–	–
Serratia marcescens ATCC 14756	–	–	–
Pseudomonas aeruginosa ATCC 27853	–	–	–
Salmonella Enteritidis ATCC 13076	–	–	–
Salmonella Typhimurium ATCC 14028	–	–	–
Proteus vulgaris ATCC 6380	–	–	–
Escherichia coli ATCC 25922	–	–	–
Acinetobacter baumannii ATCC BAA-1605	–	–	–
Enterobacter cloacae ATCC BAA-1143	–	–	–
Staphylococcus aureus ATCC 25923	+	+++	+++
Staphylococcus aureus – clinical strain 1/2017	–	–	+
Staphylococcus aureus – clinical strain 2/2017	–	+	++
Staphylococcus aureus – clinical strain 3/2017	–	+	++
Staphylococcus aureus – clinical strain 4/2017	–	–	+
Staphylococcus aureus – clinical strain 5/2017	–	–	–

* – negative; +/++/+++ weak/strong/very strong positive

ammonium sulfate had been removed. Then, 5.0 ml of washed, 1% suspension of 0,81 µm latex particles in glycine-saline buffer (pH 8.2) was added to equal volume of twice-diluted in glycine-saline buffer purified gamma globulins. The mixture was vortexed for 1 min and then allowed to incubate with gentle stirring at 37°C for 6 h. After incubation, sensitized latex particles were recovered by centrifugation, washed twice with glycine-buffered saline and finally diluted to 1% with glycine-buffered saline (pH 8.2) containing 0.1% sodium azide and 0.3% of BSA. For control, the latex reagent particles were sensitized with bovine albumin (Sigma Chemical Co., USA).

The investigated strains of *F. tularensis* (Table I) were cultured on enriched chocolate agar plates. After 48 h of incubation, a small loopful of bacteria from the strain investigated was suspended in 100 µl of PBS. The latex particles sensitized with gamma globulins and the particles sensitized with albumin bovine-control latex reagent, were parallel mixed with the same volume (25 µl) of bacterial suspensions on a black glass plate. The results were read after 1, 3 and 5 minutes of rocking the plate. Agglutination (clumping of cells) was scored as: -negative; +/++/+++ weak/strong/very strong positive. A positive latex agglutination test and a negative latex control test were confirmatory for *F. tularensis*. To assess potential cross-reactivity of the developed latex test we used the 16 different control bacterial strains as presented in Table I. The procedures of LAT with control strains were the same as for *F. tularensis*.

All manipulations with viable *F. tularensis* strains were done under biosafety level 3 (BSL-3) conditions. Keeping in mind that most diagnostic laboratories work under BSL-2 conditions, to minimize the risk of infection we also evaluated the test using inactivated bacterial suspension. For inactivation the bacterial suspension in PBS was heated at 96°C for 15 min, then cooled and used for the LAT. To verify the effectiveness of inactivation, 50 µl of the suspension were inoculated onto enriched chocolate agar plates and incubated at 37°C for 10 days.

The agglutination reactions with *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* strains after 3 minutes were very strong without any differences between live and inactivated suspensions used. We did not observe positive reactions for *F. tularensis* with the control latex reagent. No positive reactions were observed also by the LAT with *F. tularensis* subsp. *novicida* as well as with the most of control strains. However, a very strong reaction of LAT with *S. aureus*

ATCC 25923 was found after 3 minutes of rocking the plates. For this reason, we decided to investigate the additional five *S. aureus* strains isolated from hospital patients. A weak positive reaction after 3 minutes and a strong reaction after 5 minutes of rocking were observed in two cases.

Antibody coated latex particles are commonly used in diagnostic microbiology for detection, identification or serotyping of many different microbes (Miller *et al.,* 2008; Porter *et al.,* 2008; Sumithra *et al.*, 2013). In the previous work we developed the LAT for detection of antibodies against *F. tularensis* in serum samples (Rastawicki *et al.*, 2015). Here, we present the LAT for identification of *F. tularensis* that could be cultured from all kinds of samples like environmental, food, human and animal tissue samples, *etc*. In accordance with our expectation, the test recognized *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* but not *F. tularensis* subsp. *novicida*. It is because of the unique LPS composition of both subsp. *tularensis* and subsp. *holarctica,* which is different from that of *F. tularensis* subsp. *novicida* (McLendon *et al.*, 2006). The LAT recognises clinically relevant subspecies of *F. tularensis*, opposite to PCR detection of *tul4* gene, which gives positive results for all *Francisella* species and additional PCRs for other targets are necessary to differentiate *Francisella* species and subspecies (WHO, 2007). The lack of cross-reactivity of the LAT with other bacteria, except *S. aureus*, is in accordance with other researchers' results on antibody-based *F. tularensis* identification methods such as cELISA and immunochromatographic assay (Grunow *et al.,* 2000), and reveals that the test is highly specific. The cross-reactivity with some *S. aureus* strains is probably related to the presence of protein A in the cell wall of these *S. aureus* strains. It has been demonstrated that protein A expressed by some *S. aureus* strains has a high ability to bind immunoglobulins (King and Wilkinson, 1981; Romagnani *et al.*, 1981). Our experiments with other latex tests of different commercial companies, for example dedicated to detection of *Salmonella* or *E. coli* strains, also showed cross-reactivity with some *S. aureus* (data not shown). However, it is quite easy to differentiate between *F. tularensis* and *S. aureus* based on commercially available latex agglutination test for *S. aureus* or ability to grow on various microbiological media, the colonial morphology, or Gram staining. *F. tularensis* grows on rich media (enriched chocolate agar – CA, buffered charcoal yeast extract – BCYE, cystine heart agar with 9% chocolatized blood – CHAB, thioglycollate-glucose blood agar – TGBA, GC Agar II with 1% haemoglobin and 1% IsoVitaleX, sheep blood agar – SA) but does not grow on ordinary media; whereas, *S. aureus* easily grows on ordinary media such as nutrient agar (NA) and brain heart infusion agar (BHI). Also, some selective media can be applied.

The LAT developed in our study is inexpensive, simple, rapid and does not need any specialized equipment to be performed. We recommend that the results should be read after 3 minutes of rocking the plate. Thus, the test can be performed much faster compared to PCR or real-time PCR which needs at least one hour, even when the fast polymerases are used (Zasada *et al.,* 2013). Moreover, the test works well with inactivated samples which minimizes the risk of laboratory acquired infection and allows to perform the test under BSL-2 conditions. It is the important characteristic as majority of diagnostic laboratories work under BSL-2 conditions with no access to a BSL-3 laboratory. The LAT reagents shelf life is at least 2 years when stored at 4°C, as it was shown by manufacturers of commercially available latex tests for other microbes as well as our experience with in-house tests. The method is also highly reproducible between different operators (data not shown). The LAT could be an alternative for the slide agglutination test described in WHO guidelines for tularemia (WHO, 2007) when the *Francisella tularensis* antiserum is unavailable. Moreover, the use of latex particles coated with antibodies increases sensitivity of antigen detection significantly when compared to the slide agglutination test with antiserum (Drożdż, 2006).

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Literature

Eliasson H., T. Broman, M. Forsman and E. Bäck. 2006. Tularemia: current epidemiology and disease management. *Infect. Dis. Clin. N. Am.* 20: 289–311.

Formińska K., A.A. Zasada, W. Rastawicki, K. Śmietańska, D. Bander, M. Wawrzynowicz-Syczewska, M. Yanushevych, J. Niścigorska-Olsen and M. Wawszczak. 2015. Increasing role of arthropod bites in tularaemia transmission in Poland – case reports and diagnostic methods. *Ann. Agric. Environ. Med.* 22: 443–446.

Celli J. and T.C. Zahrt. 2013. Mechanisms of *Francisella tularensis* intracellular pathogenesis. *Cold Spring Harb. Perspect. Med.* 3: e010314.

World Health Organization. 2007. WHO guidelines on tularemia. WHO Library Cataloguing-in-Publication Data. WHO/CDS/EPR/2007.7, Geneva, Switzerland.

Koskela P. and A. Salminen. 1985. Humoral immunity against *Francisella tularensis* after natural infection. *J. Clin. Microbiol.* 22: 973–979.

Rastawicki W. and N. Wolaniuk. 2013. Comparison of usefulness of commercial ELISA Virion/Serion, homemade ELISA and tube agglutination test in serodiagnosis of tularemia. *Med. Dosw. Mikrobiol.* 65: 255–261.

Rastawicki W., N. Rokosz-Chudziak, A. Chróst and R. Gierczyński. 2015. Development and evaluation of a latex agglutination

test for the rapid serodiagnosis of tularemia. *J. Microbiol. Method.* 112: 1–2.

King B.F. and B.J. Wilkinson. 1981. Binding of human immunoglobulin G to protein A in encapsulated *Staphylococcus aureus*. *Infect. Immun.* 33: 666–672.

Sumithra T.G., V.K. Chaturvedi, P.K. Gupta, S.C. Sunita, A.K. Rai, M.V.H. Kutty, U. Laxmi and M.S. Murugan. 2013. Development of a simple and rapid method for the specific identification of organism causing anthrax by slide latex agglutination. *Lett. Appl. Microbiol.* 58: 401–407.

Miller R.S., L. Speegle, O.A. Oyarzabal and A.J. Lastovica. 2008. Evaluation of three commercial latex agglutination tests for identification of *Campylobacter* spp. *J. Clin. Microbiol.* 46: 3546–3547.

Porter B.D., B.D. Ortika and C. Satzke. 2014. Capsular serotyping of *Streptococcus pneumoniae* by latex agglutination. *J. Vis. Exp.* 91: e51747.

Zasada A.A., K. Formińska and K. Zacharczuk. 2013. Fast identification of *Yersinia pestis, Bacillus anthracis* and *Francisella tularensis* based on conventional PCR. *Pol. J. Microbiol.* 62: 453–455.

Drożdż R. 2006. The use of microparticles agglutination in clinical practice. *Diagn. Lab.* 42: 211–222.

McLendon M.K., M.A. Apicella and L.-A.H. Allen. 2006. Francisella tularensis: taxonomy, genetics, and immunopathogenesis of a potential agent of biowarfare. *Annu. Rev. Microbiol.* 60: 167–185.

Romagnani S., M.G. Giudizi, R. Biagiotti, F. Almerigogna, E. Maggi, G. Del Prete and M. Ricci. 1981. Surface immunoglobulins are involved in the interaction of protein A with human B cells and in the triggering of B cell proliferation induced by protein A-containing *Staphylococcus aureus*. *J. Immunol.* 127: 1307–1313.

Grunow R., W. Splettstoesser, S. McDonald, C. Otterbein, T. O'Brien, C. Morgan, J. Aldrich, E. Hofer, E.-J. Finke and H. Meyer. 2000. Detection of *Francisella tularensis* in biological specimens using capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. *Clin. Diagn. Lab. Immunol.* 7: 86–90.

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INFORMACJE Z POLSKIEGO TOWARZYSTWA MIKROBIOLOGÓW

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Konferencje Virus of Microbes dobywają się co dwa lata, a rozpoczęły się w 2010 roku w Instytucie Ludwika Pasteura w Paryżu, a następnie kontynuowane były w Brukseli (2012), Zurichu (2014), i Liverpoolu (2016), gromadząc na każdym spotkaniu ok. 400–500 uczestników z całego świata.

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VIRUSES OF MICROBES V

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KONFERENCJA MIĘDZYNARODOWA POD PATRONATEM PTM

4th Congress of Baltic Microbiologists
Gdańsk, 10–12 września 2018 r.

zapraszamy do wzięcia udziału w konferencji „4th Congress of Baltic Microbiologists",
która odbędzie się w Gdańsku w dniach 10–12 września 2018 r.

Członkowie PTM mają możliwość skorzystania ze zniżki na opłatę konferencyjną, wszelkie informacje
na stronie internetowej konferencji (informacja widoczna pod wysokościami opłat w poszczególnych kategoriach):
http://cbm2018.ug.edu.pl/general-info/fees/

Strona ze wstępnym programem konferencji – sesjami tematycznymi zawiera informację o zaproszonych wykładowcach plenarnych:
http://cbm2018.ug.edu.pl/program/

Strona z informacją o organizatorach, wykładowcach plenarnych oraz komitecie naukowym
– wszyscy członkowie komitetu naukowego będą jednocześnie zaproszonymi wykładowcami w poszczególnych sesjach tematycznych:
http://cbm2018.ug.edu.pl/general-info/committies/

Organizator:

Pan prof. dr hab. Grzegorz Węgrzyn, Uniwersytet Gdański Wydział Biologii,
ul Wita Stwosza 59, 80-308 Gdańsk.
Informacje na stronie internetowej: http://cbm2018.ug.edu.pl/

Organizatorzy wszystkich konferencji współorganizowanych przez Polskie Towarzystwo Mikrobiologów i obejmowanych patronatem PTM powinni zagwarantować zniżkowe opłaty rejestracyjne dla członków PTM (Uchwała nr 31–2017 z dnia 31.08.2017 r.)

W dniu 19.03.2018 r. odbyło się drugie zebranie
Zarządu Głównego Polskiego Towarzystwa Mikrobiologów kadencji 2016–2020

Poniżej w punktach, w formie skrótowej przedstawiono omawiane sprawy i komentarz.

1. Prezes PTM – prof. Stefan Tyski powitał zebranych – przybyli wszyscy członkowie ZG PTM (z wyjątkiem przedstawicieli Oddziału Bydgoszcz oraz Oddziału Puławy), a także 7 zaproszonych gości (dr Elżbieta Stefaniuk – przewodnicząca Głównej Komisji Rewizyjnej, prof. dr hab. Jacek Bielecki – redaktor naczelny Postępów Mikrobiologii, dr Radosław Stachowiak – z-ca redaktora naczelnego PM, Mariola Machowska-Kacprzak – księgowa PTM, dr Tomasz Zaręba, przewodniczący Sekcji Mikrobiologia Farmaceutyczna, Zbigniew Kowal, Prezes firmy Global Congress oraz Barbara Tutak z firmy Global Congress organizującej FEMS Council 2018 w Warszawie).
   Po przyjęciu programu zebrania nastąpiła prezentacja wszystkich uczestników spotkania.
2. Wybrano 2 osobową Komisję Skrutacyjną.
3. Jednomyślnie przyjęto sprawozdanie z Zebrania ZG PTM w dniu 27.03.2017 r. (Uchwała nr 12–2018).
4. Prezes PTM przedstawił informację o działalności Prezydium PTM od poprzedniego zebrania ZG PTM w dniu 27.03.2017 r.
   4.a. Poinformował o 4 internetowych zebraniach Prezydium ZG PTM w terminach: 30 sierpnia, 12 września, 31 października 2017 r. oraz 15.01.2018 r., podczas których odbyły się elektroniczne głosowania szeregu Uchwał przez członków Prezydium ZG PTM. Prezes podkreślił, że informacje o działalności PTM zamieszczane były we wszystkich zeszytach kwartalników: Postępy Mikrobiologii (PM), Polish Journal of Microbiology (PJM) oraz Medycyna Doświadczalna i Mikrobiologia (MDiM), a także w 3 listach do członków ZG PTM, wysłanych w dniach 23.05.2017 r., 13.09.2017 r. oraz 02.01.2018 r.
   4.b. Do dnia zebrania odnotowano prawie 500 „polubień" na facebooku PTM.
   4.c. Omawiając stronę internetową towarzystwa, Prezes PTM podkreślił, że została ona utworzona m.in. w oparciu o życzenia członków PTM. Zostały utworzone zakładki umożliwiające rozpoczęcie działalności szeregu sekcji PTM (bakteriologia kliniczna, wirusologia kliniczna, mykologia kliniczna, mikrobiologia farmaceutyczna, żywności, przemysłowa, środowiskowa, diagnostyka laboratoryjna), jednakże brak jest, poza sekcją mikrobiologii farmaceutycznej, zainteresowania nimi. Zwłaszcza dziwi brak aktywności diagnostów laboratoryjnych – członków PTM. Po dyskusji postanowiono jednak nie likwidować sekcji, lecz nadal umożliwić chętnym podjęcie działalności w ramach sekcji.
   Niewielki jest udział Oddziałów w rozwoju strony PTM. Co prawda zamieszczono plakaty przygotowane przez poszczególne Oddziały, z okazji konferencji „90 lat PTM", jednakże wskazane jest, aby każdy Oddział Terenowy PTM zamieszczał aktualne informacje o swojej działalności, planach, wykładach, konferencjach itp. Część Oddziałów Terenowych PTM regularnie przysyła już do biura PTM informacje wstawiane na stronę internetową. Nadal bardzo prosimy o nadsyłanie uwag, co do zawartości i wyglądu nowej strony PTM, a także podawanie informacji dodatkowych, które uważacie Państwo, że powinny dotrzeć do szerokiego gremium członków naszego Towarzystwa. (ptm.zmf@wum.edu.pl). Strona jest poprawiana i aktualizowana na bieżąco, tak, aby stać się możliwie dobrą, atrakcyjną wizytówką PTM.
   4.d. Od 1 stycznia 2018 roku utworzyliśmy możliwość logowania się na stronie PTM (do 15.03.2018 r. załogowały się 263 osoby). Strona umożliwia samodzielne uaktualnienie danych osobowych, uzyskanie informacji o płatnościach składek, a także dostęp do najnowszych numerów Postępów Mikrobiologii. Oczekujemy na propozycję, jakie jeszcze treści możemy przekazywać członkom PTM po ich załogowaniu się.

4.e. Zapoznano zebranych z informacją na temat działalności Federacji Polskich Towarzystw Medycznych, do których należy PTM, a Pani prof. dr hab. Zofia Zwolska pełni funkcję sekretarza FPTM.
Uznano, że przynależność PTM do FPTM jest korzystne dla PTM, a związane z tym płacenie rocznej składki (250 zł) jest zasadne.

4.f. Poinformowano o zakupieniu przez PTM certyfikatu SSL (Secure Socket Layer), do strony internetowej. Jest to narzędzie poświadczające wiarygodność domeny oraz jej właściciela. Potwierdza bezpieczeństwo szyfrowania danych przesyłanych pomiędzy użytkownikiem a serwerem. Jest gwarantem zachowania poufności danych i całej komunikacji. Poinformowano również o sprawach związanych z ochroną danych osobowych oraz przepisami RODO, które niedługo zaczną obowiązywać.

4.g. Poinformowano o przekazaniu w ubiegłym roku przez Fundację Polskiej Mikrobiologii na rzecz PTM kwoty 27.527 zł, w związku z likwidacją fundacji.

4.h. Poinformowano o przekazaniu w ubiegłym roku tekstu „Stanowisko Polskiego Towarzystwa Mikrobiologów w sprawie przyznania absolwentom uniwersyteckich studiów magisterskich na kierunku Mikrobiologia prawa wykonywania zawodu Diagnosty Laboratoryjnego" do Pana Prezydenta RP Andrzeja Dudy, Pani Premier Beaty Szydło, Pana Ministra ds. Zdrowia Konstantego Radziwiłła i Pana Ministra ds. Nauki Jarosława Gowina. Od Pana Ministra K. Radziwiłła otrzymaliśmy odpowiedź, że prace nad ustawą są prowadzone i nasze stanowisko będzie wzięte pod uwagę.

4.i. Przekazano informację o Nadzwyczajnym Walnym Zgromadzeniu Delegatów PTM, które odbyło się podczas konferencji „90 lat PTM". Bardzo ważną sprawą było uzyskanie opinii prawnej odnośnie możliwości powołania Pełnomocników Delegatów na NWZD PTM, co umożliwiło uzyskanie quorum na Walnym Zgromadzeniu Delegatów, wymaganego do wprowadzania zmian w Statucie PTM. Po uzyskaniu wszystkich materiałów z NWZD, z pomocą Kancelarii Prawnej został przygotowany wniosek o wpis nowego Statutu PTM do Krajowego Rejestru Sądowego (KRS), który zatwierdzi proponowane zmiany Statutu. Ze względu na drobną pomyłkę w numeracji punktów Statutu wniosek oddalono. Poprawiono Statut i ponownie złożono go do KRS – było to możliwe dzięki uchwale przyjętej podczas NWZD powołującej „Komisję Statutową do dokonania drobnych zmian w Statucie PTM na wniosek sądu".

4.j. Poinformowano o 2 wnioskach do MNiSW o przyznanie środków finansowych z puli przeznaczonej na działalność upowszechniającą naukę (DUN) – na konferencję „90 lat PTM" (otrzymaliśmy 70.000 zł, aktualnie sprawozdanie i rozliczenie finansowe konferencji oceniane jest w MNiSW) oraz na rozwój i modernizację czasopism PM i PJM w latach 2018–2019 (otrzymaliśmy informację o przyznaniu kwoty 65.200 zł)

4.k. Omówiono sprawę porządkowania listy członków PTM. Przed konferencją „90 lat PTM" usunięto z Towarzystwa ponad 200 osób nie opłacających składek członkowskich w terminie. W PTM pozostały osoby z opłaconymi składkami za lata 2015 i/lub 2016 i/lub 2017.

4.l. Prezes PTM omówił pokrótce wszystkie przyjęte przez Prezydium PTM Uchwały od czasu ostatniego posiedzenia ZG PTM (Uchwały nr 20–43 z 2017 r. i nr 1–11 z 2018 r.). Uchwały Prezydium zaakceptowano jednomyślnie (Uchwała nr 13–2018).

5. Pan prof. dr hab. Jacek Międzybrodzki, wiceprezes PTM, podsumował konferencję jubileuszową „90 lat PTM wczoraj – dziś – jutro", która odbyła się w Krakowie w dniach 22–23.09.2017 r. i oceniona była bardzo pozytywnie przez uczestników. Materiały konferencyjne znajdują się na stronie PTM, ponadto przygotowano streszczenia wykładów i plakatów w języku angielskim i opublikowano ten materiał w Suplemencie 3/2017 kwartalnika Postępy Mikrobiologii.

6. Pani dr Agnieszka Laudy, sekretarz PTM, przedstawiła listę 22 kandydatów na nowych członków zwyczajnych PTM, jednomyślnie przyjęto Uchwałę nr 14–2018 w tej sprawie. Niestety nie wpłynęła ani jedna nowa deklaracja o chęci przystąpienia do PTM członka wspierającego. Omówiła również sprawę usunięcia z grona członków PTM osób z nieopłaconą składką począwszy od roku 2015, czyli za lata 2015–2017 (zgodnie ze starym obowiązującym Statutem) – Uchwała nr 15–2018.

7. Podjęto decyzję o podniesieniu wynagrodzenia księgowej PTM z powodu dodatkowej pracy związanej z wypełnianiem formularzy dotyczących wprowadzenia jednolitego pliku kontrolnego (JPK) w księgowości (Uchwała nr 16–2018).

8. Podjęto Uchwałę nr 17–2018 w sprawie zatrudnienia Pana mgr Adama Guśpiela z NIZP-PZH na stanowisku sekretarza PJM.

9. Przychylono się do prośby Pana prof. dr hab. Grzegorza Węgrzyna z Uniwersytetu Gdańskiego o objecie patronatem przez PTM konferencji „4th Congress of Baltic Microbiologists" organizowanej przez Uniwersytet Gdański 10–12 września 2018 r. Postanowiono również poprzeć wniosek Pana profesora o dofinansowanie konferencji przez FEMS (w przypadku przyznania środków przez FEMS, PTM również dofinansuje konferencję w kwocie 250 Euro), Uchwała nr 18–2018.

10. Omówiono sprawę organizacji kolejnej edycji Konkursu o Nagrodę Naukową im. Prof. Edmunda Mikulaszka przyznawanej autorom publikacji przez PTM. Obecna edycja dotyczy prac opublikowanych w latach 2016–2017. Zwrócono uwagę na konieczność opracowania nowego regulaminu Konkursu. Regulamin zostanie opracowany przez Komisję Konkursową i zatwierdzony przez Prezydium ZG PTM (Uchwała nr 19–2018). Jednomyślnie podjęto Uchwałę nr 20–2018 o powołaniu Komisji Konkursowej w składzie: prof. dr hab. Stefania Giedrys-Kalemba, przewodnicząca oraz członkowie: prof. dr hab. Jacek Bielecki, Redaktor Naczelny kwartalnika Postępy Mikrobiologii, prof. dr hab. Elżbieta A. Trafny, Redaktor Naczelny kwartalnika Polish Journal of Microbiology, prof. dr hab. Małgorzata Bulanda, prof. dr hab. Wiesław Kaca, dr hab. Beata Anna Sadowiska, prof. nadzw. Uniwersytetu Łódzkiego, dr hab. Beata Krawczyk, prof. nadzw. Politechniki Gdańskiej.

11. Pani prof. dr hab. Ewa Augustynowicz-Kopeć, wiceprezes PTM, omówiła przygotowany przez Prezydium ZG PTM „Regulamin Działalności ZG PTM". Uchwałą nr 21–2018 Regulamin przyjęto jednomyślnie do stosowania, po zatwierdzeniu przez KRS nowego Statutu PTM.

12. Na prośbę Komisji Egzaminacyjnej przeprowadzającej Państwowy Egzamin Specjalizacyjny Diagnostów Laboratoryjnych w dziedzinie mikrobiologii medycznej dokonano wyboru nowych kandydatów reprezentujących PTM. W Uchwale nr 22–2018 przyjęto, że w Komisji Egzaminacyjnej przeprowadzającej Państwowy Egzamin Specjalizacyjny Diagnostów Laboratoryjnych w dziedzinie mikrobiologii medycznej PTM będą reprezentować: dr hab. Edyta Podsiadły, dr hab. Katarzyna Leszczyńska, dr Joanna Jursa-Kulesza oraz dr Bonita Durnaś.

13. Przewodniczący Zarządów Oddziałów Terenowych PTM przedstawili informacje z działalności swoich Oddziałów w ub. r. i okresie do 19.03.2018 r. w tym o odbytych konferencjach pod patronatem PTM. Przedstawili również plany działalności Oddziałów w najbliższych latach.

Na dzień 19.03.2018 r., składki PTM za rok 2017 i lata wcześniejsze opłaciło 413 osób, ponadto za rok **2018 składki opłaciło 501** członków PTM, odpowiednio z Oddziałów: Warszawa – 97 i **89** osób, Kraków – 60 i **64** osób, Bydgoszcz – 39 i **37** osób, Katowice – 34 i **60** osób, Szczecin – 31 i **35** osób, Poznań – 29 i **45** osób, Gdańsk – 26 i **31** osób, Lublin – 26 i **32** osób, Łódź – 19 i **26** osób, Białystok – 12 i **18** osób, Wrocław – 12 i **18** osób, Kielce – 12 i **11** osób, Puławy – 11 i **9** osób, Olsztyn – 5 i **26** osób.
Składka za 2018 r., zgodnie z prośbą ZG PTM powinna być uregulowana w I kwartale tego roku:
https://www.microbiology.pl/czlonkowie-i-skladki/czlonkowie-zwyczajni/.

14. Prezes PTM, jednocześnie delegat PTM do FEMS i IUMS, przedstawił działalność FEMS i IUMS, zwracając uwagę na uzyskane wsparcie PTM ze strony FEMS. Trzy konferencje otrzymały dofinansowanie FEMS: 90 lat PTM (3.000 Euro), konferencja międzynarodowa „Non-conventional yeast: from basic research to application", Rzeszów 15–18.05.2018 r. (5.000 Euro) oraz konferencja międzynarodowa **„**Viruses of Microbes V: Biodiversity and future applications", Wrocław, 9–13 lipca 2018 r. (5.000 Euro).
Dwaj członkowie PTM otrzymali FEMS Research Grant na wyjazdy do naukowych ośrodków zagranicznych. Niestety brak było zgłoszeń chętnych na FEMS Research Grant w I konkursie 2018 r.
Złożone zostały 2 wnioski o granty FEMS dofinansowujące wyjazdy na konferencje naukowe, jeden został zaakceptowany przez FEMS.
PTM uregulowało składki członkowskie w FEMS (5.619,85 zł) oraz IUMS (4.022,62 zł).
15. Przekazano informację o spotkaniu FEMS Council, które ma się odbyć w Warszawie w dniach 7–8.09.2018 r. Pan Zbigniew Kowal, Prezes firmy Global Congress oraz Pani Barbara Tutak poinformowali o współpracy z FEMS i ustaleniach dotyczących organizowania planowanego spotkania. Ustalono, że w przypadku dobrej organizacji FEMS Council, firma Global Congress będzie uczestniczyć w organizacji XXIX Zjazdu PTM w Warszawie we wrześniu 2020 r.
16. Pani Mariola Machowska-Kacprzak, księgowa PTM poinformowała o stanie finansowym PTM. Koszty wydawania czasopism w 2017 r. nadal były bardzo wysokie, ogólny koszt wydawania PM wyniósł – 67.093,50 zł, zaś PJM – 89.849,78 zł. Zapowiadane w 2017 r. zmiany funkcjonowania czasopism PTM w tym rezygnacja z nieodpłatnej wysyłki zeszytów PM lub PJM powinny przynieść poprawę sytuacji finansowej czasopism dopiero w bieżącym roku. Dużym wsparciem finansowym była dotacja MNiSW na Konferencję 90 lat PTM, która pozwoliła zakończyć konferencję z niewielkim zyskiem, jak również kwota przekazana przez Fundację Polskiej Mikrobiologii. Przedstawiono bilans finansowy PTM za 2017 r.
17. Pani dr Elżbieta Stefaniuk, Przewodnicząca Głównej Komisji Rewizyjnej PTM, pozytywnie skomentowała opracowany bilans finansowy PTM za 2017 r. Został on przyjęty przez członków ZG PTM i pozytywnie zaopiniowany przez Główną Komisję Rewizyjną PTM.
18. Pani Mariola Machowska-Kacprzak, księgowa PTM i Prezes PTM omówili sprawę korzystania z funduszy PTM w Oddziałach Terenowych. Problem możliwości dostępu do środków finansowych PTM i wydatkowania ich na potrzeby Oddziałów od kilku lat był podnoszony przez szereg członków ZG PTM, a także delegatów na NWZD w Krakowie. Podjęło Uchwałę nr 23–2018 w sprawie powołania komisji do opracowania regulaminu, wydatkowania i rozliczania funduszy przyznawanych Oddziałom Terenowym PTM w wysokości 10% ze składek członkowskich, jakie wpłynęły od członków PTM danych oddziałów w roku poprzedzającym wydatki. W skład komisji wejdą przedstawicie Oddziałów Terenowych ze Szczecina, Katowic i Warszawy. Komisja ta powinna opracować zasady umożliwiające Oddziałom korzystanie w 2019 r. z funduszy PTM.
Jednocześnie przypominamy, że zgodnie z Uchwałą nr 33–2017 z dnia 30.08.2017 r. Prezydium ZG PTM podjęto decyzję w sprawie udostępnienia Oddziałom Terenowym PTM od stycznia 2018 r. 50% kwoty uzyskanej z tytułu pozyskania sponsora, Członka Wspierającego PTM, darowizny, lub innej dodatkowej kwoty, na rzecz PTM, przez dany Oddział, na pokrycie kosztów prowadzenia działalności statutowej przez ten Oddział. Jak na razie Oddziały nie pozyskały żadnych dodatkowych funduszy.
19. Przekazano informacje o wydawanych czasopismach PTM i sytuacji w redakcjach:
    19.a. Pan prof. dr hab. Jacek Bielecki, Redaktor Naczelny PM, poinformował o utworzeniu nowej strony PM oraz 54 manuskryptach przesyłanych do redakcji w 2017 r. i opublikowanych 42 pracach w 4 zeszytach.
    19.b. Pani prof. dr hab. Elżbieta A. Trafny, Redaktor Naczelny PJM, poinformowała o trudnościach z prowadzeniem czasopisma PJM, rezygnacji 2 redaktorów naczelnych, prof. Izabeli Sitkiewicz i prof. Jolanty Soleckiej oraz zmianach w obsadzie redakcji. Poinformowała o rozpoczęciu wydawania internetowej wersji PJM przez firmę Exeley inc. w nowym systemie Editorial Manager. Przypomniano, że opłaty redakcyjne dla manuskryptów prac przesyłanych do PJM od 01.07.2018 r. wzrosną dwukrotnie – dla autorów korespondencyjnych, członków PTM z: 125 USD + 23% VAT do 250 USD + 23% VAT oraz dla pozostałych osób z 250 USD + 23% VAT do 500 USD + 23% VAT (Uchwała nr 40–2017).
    19.c. Przypomniano o Uchwale nr 11–2018 w sprawie niefinansowania kwartalnika Medycyna Doświadczalna i Mikrobiologia oraz rezygnacji z zapisu dotyczącego PTM w treści informacji „organ Narodowego Instytutu Zdrowia Publicznego – Państwowego Zakładu Higieny w Warszawie oraz Polskiego Towarzystwa Mikrobiologów". Jednakże informacja „W latach 1949 – 2017 kwartalnik Medycyna Doświadczalna i Mikrobiologia ukazywał się jako organ Państwowego Zakładu Higieny (obecnie Narodowy Instytut Zdrowia Publicznego – Państwowy Zakład Higieny) w Warszawie oraz Polskiego Towarzystwa Mikrobiologów" ma zostać zamieszczona na wewnętrznej stronie okładki wszystkich zeszytów kwartalnika począwszy od numeru 1/2018 r.
20. Podjęło decyzję o nie obejmowaniu patronatem PTM sesji mikrobiologicznej „Czy możliwe jest życie bez konserwantów" na międzynarodowej konferencji naukowo-szkoleniowej „Wielowymiarowość zdrowia", w terminie 12–13 października 2018 w Skierniewicach (Uchwala nr 24–2018) z powodu niedostatecznego prezentowania zagadnień mikrobiologicznych.
21. Jednomyślnie podjęto decyzję w sprawie objęcia patronatem konferencji „II Sesji Młodych Mikrobiologów Środowiska Łódzkiego" w terminie 8 czerwca 2018 roku w Łodzi (Uchwala nr 25–2018).

SEKRETARZ
Polskiego Towarzystwa Mikrobiologów
dr n. farm. Agnieszka E. Laudy

PREZES
Polskiego Towarzystwa Mikrobiologów
prof. dr hab. Stefan Tyski