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ORIGINAL PAPER

# Emergence of High-level Gentamicin Resistance among *Enterococci* Clinical Isolates from Burn Patients in South-west of Iran: Vancomycin Still Working

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Abstract

*Enterococcus faecalis* and *Enterococcus faecium* are among the main agents associated with nosocomial infections with high mortality in immunocompromised patients. Antibiotic resistance, especially against gentamicin and vancomycin among *Enterococci*, is a risk factor that could increase the morbidity and mortality rate. 179 *Enterococci* isolates from burn patients were included in this study. Antibiotic susceptibility testing was done using the disk diffusion test and minimum inhibitory concentration (MIC) was evaluated by agar microdilution. Vancomycin and gentamicin resistance associated genes including *vanA*, *vanB*, *vanC*, *aac (6')-Ie aph(2")*, *aph(3')-IIIa* and *ant(4')-Ia* were detected by PCR and their statistical relation with antibiotic resistance was evaluated. *E. faecalis* was the more prevalent strain among our local isolates and showed a higher antibiotic resistance in comparison to *E. faecium*. Vancomycin had a good antibacterial effect on the *Enterococcus* spp. isolates; however, resistance to this antibiotic and a high-level gentamicin resistance (HLGR) phenotype were observed. Among *van* operon genes, *vanA* was the most prevalent gene and among the gentamicin resistance genes, *aph (3')-IIIa* was more frequent. The HLGR *Enterococci* are a real challenge in nosocomial infections. Vancomycin is a key antibiotic to treat such infections but emergence of VRE in our region could be a real concern and, therefore, phenotypic and molecular surveillance must be considered.

Key words: *Enterococci*, gentamicin, burn, vancomycin, drug resistance

## Introduction

Burn injury is a common cause of morbidity and mortality. In Iran, approximately 30 000 people with burns present to the emergency departments each year. Among these, 3 000 dies and others either have minor burn injuries that are treated primarily in the emergency department or sustain major burn injuries that require hospital admission. Both impose the health systems a burden of cost. These numbers are eight times larger than the world average and therefore are a source of concern (Karimi et al. 2015).

In most cases, the bacterial infection of burn wounds is an unquestionable phenomenon because of the skin destruction, which plays a role of the major barrier to bacterial access to the internal tissues. Gram-positive bacteria including *Staphylococcus aureus*, β-hemolytic *Streptococci* and *Enterococci*, and Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacteriaceae* are among the most frequent etiological agents of burn patients infections (Norbury et al. 2016).

The facultative anaerobic Gram-positive *Enterococcus* spp. normally colonize the gastrointestinal tract, oral cavity, and vaginal tract. *Enterococci* are among the major agents associated with nosocomial infections particularly in burn patients presenting with bacteremia, urinary tract infections and endocarditis (Hashem et al. 2017). The US National Nosocomial Infection Surveillance (NNIS) system, has ranked *Enterococci* among

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the top three most common pathogens of nosocomial infections and the leading cause of nosocomial infections in burn patients (Pan 2012).

*Enterococci* are intrinsically resistant to multiple antibiotic agents: cephalosporins, penicillinase-resistant penicillins, and low concentrations of aminoglycosides (Hollenbeck and Rice 2012). Vancomycin remains the drug of choice to treat enterococcal infections but nowadays vancomycin-resistant *Enterococci* (VRE) isolates are a global challenge. Such infections are treated by a combination of cell wall-active agents with aminoglycosides that achieve synergistic bactericidal activity. Aminoglycoside antibiotics are positively charged, carbohydrate-containing molecules that find a clinical use for the treatment of infections caused by both Gram-negative and Gram-positive bacteria (Miller et al. 2014). Two antibiotic resistance phenotypes among *Enterococci* including VRE and HLGR have emerged as important nosocomial causes throughout the world as well as in Iran (Sakoulas et al. 2013a; Emaneini et al. 2016; Osuka et al. 2016). Operon associated genes (*van* operon) are responsible for VRE emergence and are the best studied antibiotic resistance operon (Faron et al. 2016).

In contrast to the rest of aminoglycosides, gentamicin is not inactivated by target insensitivity and enzymatic modification and, therefore, remained for many years the aminoglycoside often used to achieve synergistic killing of *Enterococci*. However, there are some variants of aminoglycosides modifying enzymes (AMEs) that can affect gentamicin (Garneau-Tsodikova and Labby 2016).

There are limited studies regarding the simultaneous resistance to vancomycin and gentamicin in hospitalized burn patients in Iran and thereby, we aimed to follow up and monitor the antibiotic resistance pattern and the genes encoding resistance to these two antibiotics among *Enterococcus* spp. strains isolated from burn patients.

## Experimental

### Materials and Methods

**Bacterial isolates.** One hundred seventy-nine out of 628 bacterial isolates confirmed as *Enterococcus* spp. were isolated from different clinical specimens (wound biopsies and blood) of burned patients referred to Taleghani burn hospital (the only referral burn center in Ahvaz, Khuzestan province, Iran) during January 2015 to 2016. All isolates were primary identified by conventional microbiological methods and confirmed by specific tests such as bile esculin hydrolysis and PYR (Pyrrolidinyl Aminopeptidase) Test. Sugar fermentation (arabinose and sorbitol) were used for characterization of *Enterococcus* species (Emaneini et al. 2016). Molecular confirmation of the species was done by screening for the $ddl_E$ (D-alanine-D-alanine ligase) gene (Dutka-Malen et al. 1995).

**Antimicrobial susceptibility testing.** Susceptibility to antimicrobial agents was determined by disk diffusion method according to CLSI criteria using commercially available disks (Mast, UK) including vancomycin, teicoplanin, gentamicin, chloramphenicol, linezolid, ciprofloxacin, and amoxicillin. The Minimum Inhibitory Concentration (MIC) against gentamicin and vancomycin were determined using two-fold serial agar dilution method with Mueller Hinton agar (Difco, USA) according to CLSI guidelines (CLSI 2015). *E. faecalis* ATCC 29212 and *E. faecium* IP 4107 (The Collection of Institut Pasteur, France) were used as quality control reference strains.

**DNA extraction and PCR.** DNA was extracted using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea). The oligonucleotide primers used in this study are listed in Table I. For each sample, the PCR assay was performed to identify *vanA, vanB, vanC* genes for vancomycin resistance and *aac(6')-Ie aph(2")*, *aph(3')-IIIa* and *ant(4')-Ia* genes for gentamicin

Table I
Primers used in this study.

| Target gene | Oligonucleotide sequences (5'-3') | Size of product (bp) | Reference |
|---|---|---|---|
| ***van A*** | GGGAAAACGACAATTGC<br>GTACAATGCGGCCGTTA | 732 | Dutka-Malen et al. 1995 |
| ***vanB*** | ATG GGA AGC CGA TAG TC<br>GAT TTC GTT CCT CGA CC | 638 | Dutka-Malen et al. 1995 |
| ***vanC*** | AAT CGT CAA TTC CTG CAT GT<br>TAA TCG TGG AAT ACG GGT TTG | 299 | Dutka-Malen et al. 1995 |
| ***aac(6')-Ie aph(2")*** | AGGAATTTATCGAAAATGGTAGAAAAG<br>CACAATCGACTAAAGAGTACCAATC | 369 | Vakulenko et al. 2003 |
| ***aph(3')-IIIa*** | GGCTAAAATGAGAATATCACCGG<br>CTTTAAAAAATCATACAGCTCGCG | 523 | Vakulenko et al. 2003 |
| ***ant(4')-Ia*** | CAAACTGCTAAATCGGTAGAAGCC<br>GGAAAGTTGACCAGACATTACGAACT | 294 | Vakulenko et al. 2003 |

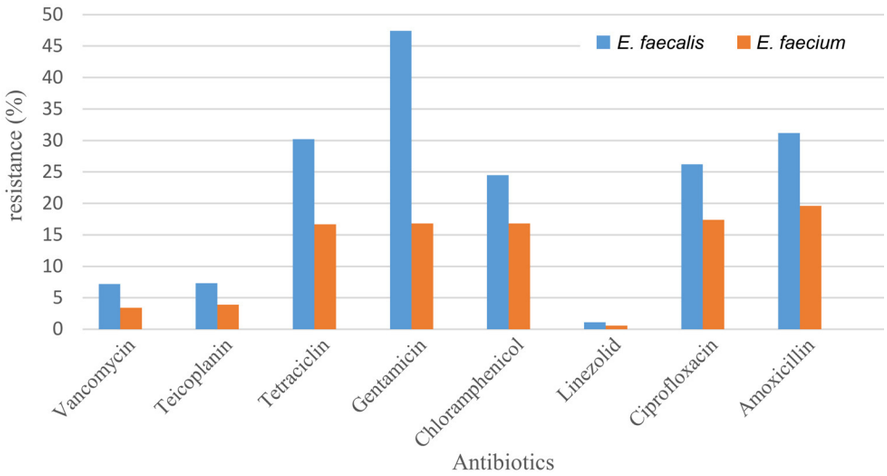


Fig. 1. Antibiotic resistance pattern of *E. faecalis* and *E. faecium* isolated in this study against eight antibacterial agents.

resistance. DNA amplification was carried out in a peqSTAR thermal cycler system using a defined protocol as described previously (Dutka-Malen et al. 1995; Vakulenko et al. 2003).

**Statistical analysis.** We used absolute and relative frequency to present descriptive statistics. Chi-Square test and also Fisher exact test (if it was necessary) used to explain analytical statistics. Data were analyzed by SPSS version 22.

## Results

The patients referred to the hospital had different degrees of burn. From 628 bacterial isolates, isolated from patient referred to our hospital, 28.5% (179 isolates) were confirmed as *Enterococci* and among them 108 (60.3%) were *Enterococcus faecalis* and 71 (39.7%) were *Enterococcus faecium*. These species were isolated from blood (29.6%) and wound (70.4%) of burn patients. The drug resistance pattern of the isolates against eight antibiotics including chloramphenicol, linezolid, ciprofloxacin, amoxicillin, teicoplanin, vancomycin, and gentamicin is shown in Fig. 1. Overall, drug resistance of *E. faecalis* was significantly higher than that of *E. faecium* ($p < 0.03$). Among the aminoglycosides, the highest resistance rate was against gentamicin, and was observed in 45% of the *E. faecalis* and 15% of the *E. faecium* isolates, respectively. The prevalence of resistance to amoxicillin and tetracycline came in the second and third positions. However, a good sensitivity was observed toward vancomycin.

The frequency of vancomycin resistance associated genes: *vanA*, *vanB*, *vanC* and gentamicin resistance associated genes: *aph*(*3'*)-*IIIa*, *ant*(*4'*)-*Ia* and *aac*(*6'*)-*Ie aph*(*2'*) in the two *Enterococcus* species is reported in Table II.

The *vanA* gene was the most frequent vancomycin associated gene and it was detected in 23 positive cases (12.8%). As shown in Table II, the *vanA* and *vanB* genes were detected simultaneously in 6 isolates. The vancomycin MICs among VRE isolates ranged between 64 mg/l and 1024 mg/l. There was not a significant correlation between the presence of *van* operon genes and the vancomycin MICs.

Among the gentamicin resistance genes, the highest frequency was observed for *aph*(*3'*)-*IIIa* in 68% (n = 122) and *aac*(*6'*)-*Ie aph*(*2'*) in 61% (n = 109) of the cases.

The prevalence of *aph*(*3'*)-*IIIa, ant*(*4'*)-*Ia* and *aac*(*6'*)-*Ie aph*(*2'*) in HLGR isolates were 10.8%, 65.5% and 60.1% respectively.

The simultaneous presence of at least two gentamicin resistance associated genes also were observed

Table II
Frequency of vancomycin and gentamicin resistance genes among our local *Enterococci* isolates.

| Genus | *van* gene | | | | Gentamicin gene | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *vanA* | *vanB* | *vanA, vanB* | *vanC* | *3'* | *4'* | *6'* | *3'+4'* | *3'+6'* | *4'+6'* | *3'+4'+6'* |
| *E. faecalis* | 14 | 3 | 3 | 1 | 74 | 8 | 67 | 3 | 26 | 6 | 4 |
| *E. faecium* | 9 | 0 | 3 | 2 | 48 | 10 | 42 | 6 | 50 | 2 | 1 |
| Total (*n*) | 23 | 3 | 6 | 3 | 122 | 18 | 109 | 9 | 76 | 8 | 5 |

Abbreviations are as follows: *3: aph* (*3'*)-*III;, 4': ant* (*4'*)-*I;, 6': aac* (*6'*)-*Ie aph* (*2"*)

Table III
The association between presence of gentamicin resistance genes and MICs against gentamicin.

| Gene target | | MIC (μg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 256 | | 512 | | 1024 | |
| | | *E. faecalis* | *E. faecium* | *E. faecalis* | *E. faecium* | *E. faecalis* | *E. faecium* |
| **Gentamicin resistance gene** | *aph(3')-IIIa* | 0 | 0 | 3 | 1 | 2 | 3 |
| | *ant(4')-Ia* | 0 | 0 | 3 | 1 | 0 | 0 |
| | *aac(6')-Ie aph(2")* | 0 | 0 | 5 | 2 | 2 | 2 |

and the most frequently, the simultaneous presence of *aph*(*3'*)-*IIIa* and *aac*(*6'*)-*Ie aph*(*2'*) genes wad detected on 109 cases (60.8%). There was no significant correlation between aminoglycoside resistance emergence and the prevalence of these genes.

The relation between the aminoglycoside resistance genes and the MIC of aminoglycosides antibiotics is reported in Table III. Of 122 isolates that were harboring the *aph*(*3'*)-*IIIa* gene, 7.3% (n = 9) had the gentamicin MIC of ≥ 512 μg/ml. The gentamicin MICs for isolates harboring *ant*(*4'*)-*Ia* and *aac*(*6'*)-*Ie aph* (*2'*) were 3 μg/ml and 11 μg/ml respectively.

The HLGR phenotype was reported in 82.7% of the isolates in this study (57.4% of *E. faecalis* and 42.6% of *E. faecium* isolates, respectively). There was no significant correlation between HLGR rate and the species of *Enterococci* ($p = 0.2$).

## Discussion

We studied 179 *Enterococci* isolates from patients, which were referred to a burn center in Ahvaz, southwest of Iran and this number is higher than other similar studies that could be a point for our study. The morbidity and mortality associated with nosocomial infections due to antimicrobial resistant *Enterococci* demonstrated a crude mortality rate of 17–100% in case of enterococcal bacteremia in different hospitals around the world (Edmond et al. 1996).

The clinical importance of *Enterococci* is directly related to their antibiotic resistance, which contributes to the risk of colonization and infection. The species of the greatest clinical importance are *E. faecalis* and *E. faecium* (Kajihara et al. 2015). In our study, *E. faecalis* was more prevalent, which is in concordance with other reports (Olawale et al. 2011; Komiyama et al. 2016).

The increase of antimicrobial resistance among *Enterococcus* spp. is a serious health problem globally and there are several reports of antimicrobial resistance among *Enterococci* isolated from hospitalized patients in Iran and other countries (Emaneini et al. 2016; Lan et al. 2016). In this study, antibiotic resistance rate in *E. faecalis* isolates was significantly higher than *E. faecium*. However, it has been reported that notwithstanding of its lower frequency, *E. faecium* has a more ability to develop antibiotic resistance (Werner et al. 2008).

Although there are an increasing number of reports on VRE emergence in other countries, in the present study most of the isolates were susceptible to vancomycin. This could be due to lower usage of vancomycin in the first place because treatment is done with other antibacterial agents. Moreover, the VRE phenotype is more often associated with *E. faecium* (Werner et al. 2008; Arias et al. 2010), which was not prevalent in our study. The *vanA* and *vanB* genes are the most frequently vancomycin resistance associated genes among *Enterococcus* spp. *Enterococci* which harbor the *vanA* gene, are resistant to vancomycin (MIC ≥ 64 μg/ml) and teicoplanin (MIC ≥ 8 μg/ml) at a high concentration. Resistance is induced by the presence of these drugs (Eliopoulos and Gold 2001). In our study the *vanA* gene was more prevalent but totally the vancomycin resistance was observed only in 10.6% of the isolates, which accordingly to the ability of the resistance induction in the presence of the antibiotic, could be associated with lack of exposure to vancomycin and teicoplanin, as it was mentioned previously. The *vanB* harboring *Enterococci* are resistant to a range of vancomycin concentrations: from 4 to over 1024 μg/ml. Such strains remain susceptible to teicoplanin. The *vanC* gene was reported in *E. gallinarum* and *E. casseliflavus*, which are intrinsically resistant to vancomycin at concentrations typically lower than or equal to 32 μg/ml (Eliopoulos and Gold 2001). In this study, we observed the *vanC* gene in three isolates and this gene could has been transmitted from these organisms to the clinically important *E. faecalis* and *E. faecium* spp.

In addition to the costs imposed to health systems, the importance of the VRE emergence is that these strains could serve as a *van* genes reservoir for other organisms, especially *Staphylococcus aureus*. This could be a real problem because vancomycin is the therapeutic agent of choice for methicillin-resistant *S. aureus* (Gardete and Tomasz 2014).

For the first time, the HLGR phenotype was reported in *E. faecalis* in 1979 in France, followed by some U.S. healthcare institutions, in which 25% of *E. faecium* iso-

lates displayed the HLGR phenotype and a decade later, more than 60% of *E. faecium* isolates demonstrated this phenotype (Kobayashi et al. 2003).

Nine genes that encode enzymes targeting eight different aminoglycosides have been identified. In most cases, the *aac(6')-Ie aph (2')* gene has been found to be associated with resistance to aminoglycosides (Ramirez and Tolmasky 2010).

Like in this study, the HLGR was reported high in other similar studies from Iran, (Emaneini et al. 2016; Heidari et al. 2017). The studies conducted in other countries have also been reported a high rate of the HLGR phenotype. Almost in all these studies, high rate of HLGR was more prevalent in *E. faecalis* which could be related to its higher prevalence in the clinic (Wendelbo et al. 2003; Adhikari, 2010). Thus, the prevalence of *aph*(*3'*)*-IIIa, ant*(*4'*)*-Ia* and *aac*(*6'*)*-Ie aph*(*2'*) was higher in *E. faecalis* isolates in comparison to *E. faecium* isolates.

We could not find any report that study the relation between the presence of aminoglycosides resistance genes and the HGLR rate. The prevalence of the HLGR phenotype and the aminoglycosides resistance genes varied in the studies from all over the world (Udo et al. 2004). Nowadays, there are several genes conferring aminoglycosides resistance among *Enterococci* including the *aph*(*2*")*-Ic, aph*(*2*")*-Id, aph*(*2*")*-Ib genes*, and the *aac*(*6'*)*-Ie-aph*(*2'*)*-Ia* gene is no longer the only gentamicin resistance gene (Chow et al. 1997; Tsai et al. 1998; Kao et al. 2000). Almost all these genes are located on transposable elements, which contribute to their easy dissemination and this poses a challenge for health systems. Treatment of HLGR *Enterococci* is a real problem. Recently, few reports suggested using an anti-peptidoglycan active agent in combination with a membrane active agent. Accordingly, a successful treatment of endocarditis caused by *E. faecalis* of HLGR phenotype was demonstrated after administration of daptomycin plus ceftaroline (Sakoulas et al. 2013b). Although the HLGR *Enterococci* are predominant in our region, vancomycin keeps its antimicrobial effect on such strains. However, we reported VRE isolates and this could be a "red alarm" to our health system, and thus a continuous surveillance using both genetically and phenotypically methods should be done for this type of resistance.

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**Conflict of interest**

The authors have no financial conflicts of interest.

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ORIGINAL PAPER

# *Aspergillus penicillioides* Speg. Implicated in Keratomycosis

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Abstract

The aim of the study was mycological examination of ulcerated corneal tissues from an ophthalmic patient. Tissue fragments were analyzed on potato-glucose agar (PDA) and maltose (MA) (Difco) media using standard laboratory techniques. Cultures were identified using classical and molecular methods. Macro- and microscopic colony morphology was characteristic of fungi from the genus *Aspergillus* (restricted growth series), most probably *Aspergillus penicillioides* Speg. Molecular analysis of the following rDNA regions: ITS1, ITS2, 5.8S, 28S rDNA, LSU and β-tubulin were carried out for the isolates studied. A high level of similarity was found between sequences from certain rDNA regions, i.e. ITS1-5.8S-ITS2 and LSU, what confirmed the classification of the isolates to the species *A. penicillioides*. The classification of our isolates to *A. penicillioides* species was confirmed also by the phylogenetic analysis.

Key words: *Aspergillus penicillioides*, morphology, genetic characteristic, cornea

## Introduction

Fungi from the genus *Aspergillus* are anamorphic stages of ascomycetes *Ascomycota*, class *Eurotiomycetes,* order *Eurotiales* and family *Trichocomaceae* (syn. *Eurotiaceae*) (Kirk 2008). These are the fungi with a high morphological and genetic variability (Thom and Raper 1945; Peterson 2000, 2003).

They are most often saprotrophs or facultative parasites (Agrios 2005). They play a significant role in consumable and pharmaceutical industries. These fungi occur on plants, in soil, animal remnants, storage products or public utility places (Thom and Raper 1945; Ejdys 2007; Krijgsheld et al. 2013; Ogar et al. 2015). They are quite common in different climate zones, especially in subtropical and tropical regions (Thom and Raper 1945; Krijgsheld et al. 2013). These fungi are adapted to grow in high temperatures and a warm climate (Gock et al. 2003). They are the causative agents behind diseases such as aspergilloses (Bossche et al. 1988; Seyedmousavi et al. 2015). The aspergilloses of the human respiratory system, skin, ears, sinuses, especially in patients with immunodeficiency or cystic fibrosis has already been reported (Bossche et al. 1988; Sandhu et al. 1995; Hamilos 2010; Gupta et al. 2015; Walicka-Szyszko and Sands 2015).

Aspergilloses have been observed in bees, domestic birds and farm animals (Puerta et al. 1999; Lugauskas et al. 2004; Seyedmousavi et al. 2015). Many species produce secondary metabolites that are toxic to humans and warm-blooded animals. Aflatoxins and their derivatives ($B_1$, $B_2$, $G_1$, $G_2$, $M_1$, $M_2$) are *Aspergillus* spp. mycotoxins that are strongly poisonous to humans and animals. Aflatoxin $B_1$ is considered the most carcinogenic substance (Agrios 2005). Ohratoxin A, sterygmatocistin and patulin are among other substances toxic to humans and animals (Bayman et al. 2002).

There is more and more information in the literature about ocular mycoses (Mendiratta et al. 2005; Mitani et al. 2009; Machowicz-Matejko and Zalewska 2015). Different taxa of fungi could be the cause of these infections i.e.: *Candida*, *Colletotrichum*, *Fusarium*, *Penicilllium*, *Aspergillus* and other (Sharma et al. 1993; Gunawardena et al. 1994; Thomas 2003; Chowdhury and Singh 2005; Nayak 2008). *Aspergillus* spp. are among the most frequently isolated fungi from the cornea (Nayak 2008).

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Ocular mycoses are very difficult to eliminate, and it is essential to determine the aetiology of infection in order to select the proper treatment.

The aim of the study was the mycological examination of the affected corneal tissue from a patient with corneal ulceration.

## Experimental

### Materials and Methods

**Material.** The research material consisted of a fragment of the cornea with deep ulcer and inflammatory infiltration taken during the "on hot" surgery of the corneal transplant from the patient in the Department of Diagnostics and Microsurgery of Glaucoma at the Medical University in Lublin (Fig. 1).

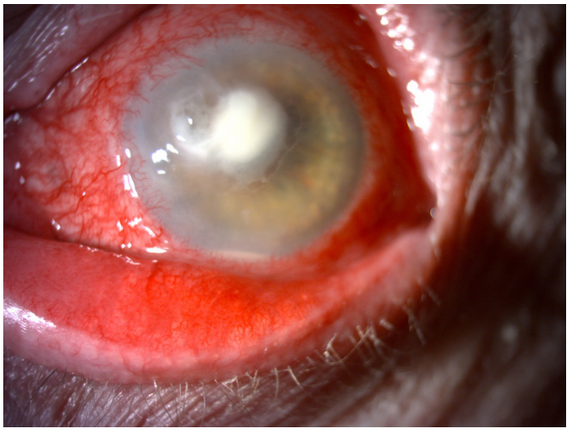

Fig. 1. The disease symptoms of the cornea from which *A. penicillioides* was isolated.
Photo E. Machowicz-Matejko.

**Patient Characteristics.** An 84-year-old man was treated for pneumonia a few months ago before his admission to the hospital. Moreover, he was also treated for diabetes, hyperthyroidism, atherosclerosis and kidney disorder. This patient was hospitalized because of severe keratitis of unknown aetiology. Cornel scrubs and cultures were taken for the microbiological analysis on different culture media. The negative results for bacteria and fungi were obtained after seven days of culture on the appropriate media. In spite of the empirical treatment with antibiotics and antifungal agents the corneal inflammation continued to be very severe. After nine days of local and systemic treatment we decided about corneal transplantation "on hot". The tissue of the cornea was taken to the microbiological examination. After the seven days of the culture on PDA and MA culture media, using standard laboratory methods (FAO 2006) no colonies were visible on the media. Despite on this we decided to carry on our analysis for a longer time.

The research material was kept in a physiological solution of 0,9% NaCl at room temperature. The mycological analysis of cornea pieces was carried on PDA and MA culture media, using standard laboratory techniques.

Two isolation methods were used: the first (I) – 0.5 mm cornea fragments were placed on solidified culture media in Petri dishes – 4 pieces per one dish; and the second (II) – suspension of the fungus hyphae that passed from the cornea to the physiological solution was transferred to sterile Petri dishes in a volume of 0.1 ml and 0.5 ml, and poured over a chilled liquid PDA (own method). The samples were then incubated in a thermostat at a temperature of 25°C. The visible colonies of fungus were transferred to slants with PDA medium and subsequently single spore cultures were prepared and allowed to grow and develop. The cultures were identified using classical and molecular methods.

The species name and author's initials were corrected in the current status of the Index Fungorum taxonomic species database in 2016 and according to Sklenář et al. (2017) elaboration.

**Classical identification.** Pure fungus cultures were placed as a small inoculum on agar media in Petri dishes, using three inocula per one Petri dish, which was then sealed with a parafilm to prevent drying. Five different culture media were used: Czapek-Dox, PDA, Sabouraud, YPD (Thom and Raper 1945; FAO 2006) and a poor medium (5 g – glucose, 20 g – agar, 1 $dm^3$ – distilled water). The cultures of fungus were carried out at a temperature of 25°C for one month. Considering the very slow growth of fungi, the temperature was raised to 30°C and the period of incubation was extended.

Macroscopic and microscopic observations and photographic documentation were performed from the start of visible colony growth, i.e. from day 17 of culture. Photographs of the morphological structures using a Scanning Electron Microscope (SEM) (Tescan Vega/LMU) were taken at the Central Laboratory of Agro-Ecological (CLA), the University of Life Sciences in Lublin.

**Molecular identification.** The research material used in this study consisted of 3 different isolates, obtained from the human cornea (Nos 1–3). The colonies prepared for DNA isolation were grown on PDA medium. Identification of the isolates to the species level was carried out using universal primers for rDNA genes: the ITS1-5.8S-ITS2 – (ITS1 and ITS4) region, 28S rDNA region – LSU – (LR5 and LROR) and for the β-tubuline – gene ($Bt_2a$ and $Bt_2b$) (Table I).

**DNA isolation.** Genomic DNA isolation was performed using the CTAB method by Doyle and Doyle (1987) with some own modifications. Fungus isolates

Table I
Primers used in PCR reaction.

| Name | Primer | Primer sequence 5'-3' | Melting temperature (Tm) °C | Primer orientation | Reference |
|---|---|---|---|---|---|
| ITS primers | ITS1 | TCCGTAGGTGAACCTGCGG | 59 | Forward | Ref. 28 |
| | ITS4 | TCCTCCGCTTATTGATATGC | 59 | Reverse | Ref. 28 |
| LSU primers | LROR | ACCCGCTGAACTTAAGC | 60 | Forward | Ref. 28 |
| | LR5 | TCCTGAGGGAAACTTCG | 60 | Reverse | Ref. 29 |
| β-tubulin primers | $Bt_2a$ | GGTAACCAAATCGGTGCTGCTTTC | 60 | Forward | Ref. 30 |
| | $Bt_2b$ | ACCCTCAGTGTAGTGACCCTTGGC | 60 | Reverse | Ref. 30 |

were grown at a temperature of 27°C for 45 days. The obtained mycelium was transferred to Eppendorf tubes of a 1.5 ml volume, and then frozen in liquid nitrogen. The frozen material was homogenized with a sterile pestle (Sigma-Aldrich) until mycelium homogeneity. Subsequently, 600 ml of CTAB lysis buffer was added to each tube and incubated at 65°C for 2 hours and then centrifuged (10 000 rpm for 10 minutes). In the next step, 1.0 ml phenol/chloroform/alcohol mixture in the volume ratio of 25:24:1 was added to the supernatant and centrifuged (10 000 rpm for 8 minutes). DNA was precipitated with 40 µl of sodium acetate (5 M) and 400 µl of isopropanol. The resulting precipitate was washed with 70% ethanol, and then centrifuged at 14 000 rpm for 15 minutes and dried. The supernatant was suspended in 50 µl of PCR water (Sigma-Aldrich), supplemented with RNase A, and then incubated for 25 hours at a temperature of 4°C, followed by one hour at 37°C, and 10 minutes at 65°C. DNA was run in 1.5% agarose gel electrophoresis and compared with GeneRuler 100 bp DNA Ladder Plus marker (Thermo Scientific). The DNA quantity was measured with a UV-Vis NanoDrop 2000c/2000 spectrophotometer (Thermo Scientific). Then, the DNA samples were diluted to a final concentration of 50 ng/µl and stored at 20°C for further analysis.

**PCR amplification and sequence analysis.** Three different primers were used for identification of *Aspergillus* spp. isolates: ITS1 and ITS4, LROR and LR5, and $Bt_2a$ and $Bt_2b$ (Table I). The amplification reaction volume was 25 µl and the reaction solution contained 20 ng/µl of genomic DNA, 1 × Buffer *Tag* (750 mM Tris HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20) (Thermo Scientific), 0.1 mM × $MgCl_2$, dNTP mix 1 mM of each primer 0.2 µM and 1.0 U polymerase DNA (Fermentas). Amplification reaction was performed using a DNA Engine Dyad Thermal Cycler (Biorad). The PCR amplification reaction for ITS1 and ITS4 primer pair was carried out using the following program: initial denaturation for 5 min at 95°C, followed by 40 cycles consisting of 30 s at 95°C, 50 s at 57°C, 60 s at 72°C and final extension for 10 min at 72°C. PCR reaction programs for two primers pairs, the first, LROR and LR5, and the second, $Bt_2a$ and $Bt_2b$, were subjected to minor changes, as follows: initial denaturation for 5 min at 94°C, then 36 cycles of annealing: 30 s at 94°C, 50 s at 56°C, 55 s at 72°C and final extension of the product at 72°C for 5 min. The obtained products were separated by electrophoresis using 1.5% agarose gel containing 7.5 µl of ethidium bromide in 1 × TBE buffer at 80 V for 1.5 hour in the presence of *GeneRuler* 100 bp Plus DNA Ladder (Thermo Scientific). The PCR products were further sequenced using Applied Biosystems BigDay® Terminator v. 3.1. kit (Life Technologies) and separated on a 3730xl DNA Analyser capillary sequencer. The nucleotide sequences obtained were compared with the nucleotide sequence deposited in GenBank at the National Centre for Biotechnology Information NCBI (http://www.ncbi.nlm.nih.gov/), using the Mega Blast program (Zhang et al. 2000; Morgulis et al. 2008). The "local sequence alignment" method was used as a comparison criterion. The highest degree of sequence identity was used to determine the species of the isolates. The phylogenetic analysis of ITS sequences of our three isolates of *A. penicillioides* studied was carried out in several steps using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Moreover, 13 other reference strains of four *Aspergillus* spp. i.e.: *A. salinicola* – strains EXF_10401, IBT_34266, KAS_6054, *A. hordei* – NRRL25826, NRRL25825, NRRL25830, *A. clavatophorus* – DTO257-G5, NRRL_25874 and 5 strains of *A. penicillioides* (mentioned in Table II) were also included. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated.

Table II
The degree of identity and length of the covered sections of sequences as defined by the BLAST methods.

| Species | Strain/isolate | The analyzed sequences from GenBank accession No. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ITS | | | LSU | | | β-tubulin | | |
| | | No. GenBank | The degree of identity of sequence [%] = identity | Query cover nucleo-tides | No. GenBank | The degree of identity of sequence [%] = identity | Query cover nucleo-tides | No. GenBank | The degree of identity of sequence [%] = identity | Query cover nucleo-tides |
| *Aspergillus penicillioides* | isolate B1-8 | KF986414.1 | 100 | 569 | | | | | | |
| *Aspergillus penicillioides* | UFMGCB 6310 | KF373543.1 | 100 | 498 | | | | | | |
| *Aspergillus* sp. | DY115-21-7-M6 | KF411572.1 | 100 | 551 | | | | | | |
| Uncultured fungus | clone LX042233-122-012-B01 | GQ999240.1 | 99 | 574 | | | | | | |
| *Aspergillus penicillioides* | isolate KH00279 | GU017500.1 | 99 | 574 | GU017541.1 | 99 | 835 | | | |
| *Aspergillus penicillioides* | isolate KH00251 | GU017494.1 | 99 | 574 | GU017535.1 | 100 | 833 | | | |
| Uncultured fungus | clone PR-MAT-CV5-17 | FJ265955.1 | 99 | 574 | | | | | | |
| *Aspergillus* sp. | F55 | FJ755819.1 | 99 | 574 | | | | | | |
| *Aspergillus penicillioides* | strain WR1996 | KP997215.1 | 99 | 544 | | | | | | |
| *Aspergillus penicillioides* | Culture collection CCF<CZE> 3112 | FR727125.1 | 99 | 573 | | | | | | |
| Uncultured fungus | clone PR-MAT-CV5-19 | FJ265957.1 | 99 | 571 | | | | | | |
| *Aspergillus penicillioides* | isolate HNC15-78 | KT959298.1 | 99 | 530 | | | | | | |
| Uncultured fungus | clone xnh90 | KP063524.1 | 99 | 574 | | | | | | |
| *Aspergillus* sp. | 43m | KC834810.1 | 99 | 527 | | | | | | |
| Uncultured *Aspergillus* | clone CHiv38 | KP974191.1 | 99 | 574 | | | | | | |
| Uncultured fungus | clone xnh20 | KP063454.1 | 99 | 574 | | | | | | |
| Uncultured *Aspergillus* | clone Leof80 | KF225869.1 | 99 | 574 | | | | | | |
| *Aspergillus penicillioides* | clone KJ34-0.2-39 | | | | KT390118.1 | 99 | 791 | | | |
| *Aspergillus penicillioides* | | | | | U81265.1 APU81265 | 99 | 783 | | | |
| *Aspergillus* sp. | CCF 3112 | | | | | | | FR775323.1 | 97 | 506 |

There was a total of 455 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

## Results

Pathological changes in the eye involved the cornea. There were superficial epithelial changes and alterations in deep stromal layers. Corneal ulceration, 7 mm in diameter, was surrounded by a white infiltration and endothelial deposits were observed. Corneal tissue at the site of ulceration was completely opaque, while surrounding tissue was cloudy because of the inflammation (Fig. 1). Two fungal colonies were cultured from the examined tissues using method I and several colonies using method II of fungus isolation. The first colonies appeared only just after 15–17 days of incubation on PDA. There was no growth on MA medium regardless of the method of isolation.

**Morphological characteristics.** A characteristic feature of the studied fungus was extremely limited growth of the colonies. After 1.5 months on Czapek-Dox medium, the diameter of the colony ranged from 0.8 to 1.0 cm, on PDA from 1.2 to 1.6 cm, and on YPD medium and Sabouraud from 1.2 to 1.4 (Fig. 2). On the poor medium, the growth of colony was hardly noticeable. Larger diameters of the colonies were observed after 2–2.5 months of culture.

The colonies on Czapek-Dox, PDA, Sabouraud and YPD medium were light-beige in color, slightly darkening with age, while on poor medium they were white-grey (Fig. 2). The reverse of the colonies was bright to dark beige. An olive color of the colonies appeared only after two months of growth on Sabouraud medium.

Surface of the colonies was velvety, compact and strongly wrinkled with radial grooves, appearing after a long time of culture. Only on a poor medium, the colonies were flat (Fig. 2).

In microscopic slights of mycelium taken from Czapek-Dox, PDA, Sabourauda and YPD culture media, numerous conidiophores with smooth walls, growing from substrate mycelium or aerial mycelium,

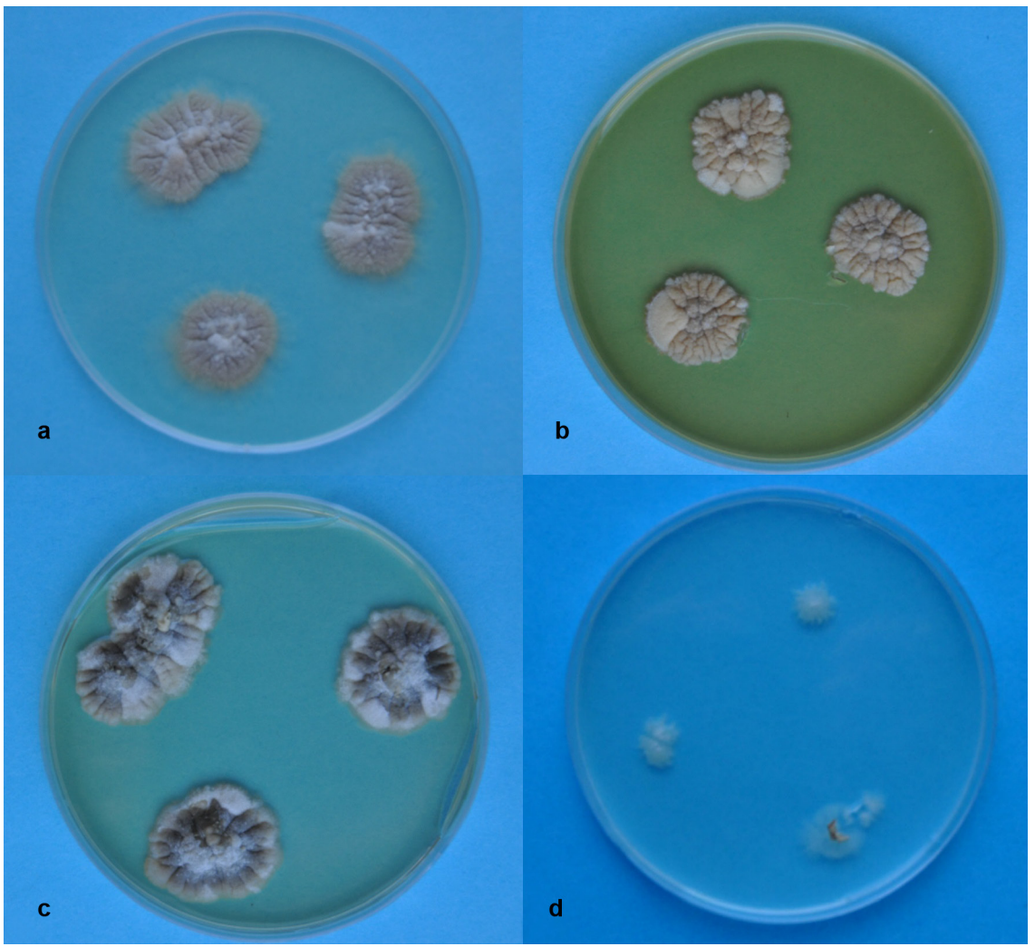


Fig. 2. Colonies of *A. penicillioides* after 8 weeks of growing on culture media.
a – PDA, b – YPD, c – Sabouraud, d – a poor medium (see M&M section). Photo E. Zalewska.

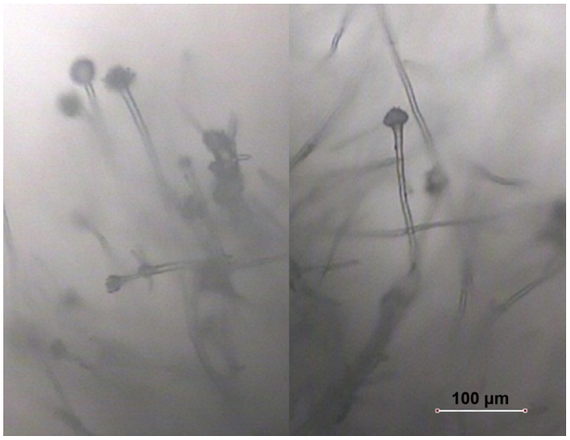


Fig. 3. *A. penicillioides* cultured on PDA.
Photo E. Zalewska.

were observed (Fig. 3). Conidiophores contained oval or flask-shape vesicles (Figs. 4, 5). Four to several phialides grew from the upper part of vesicles in single row (Figs. 4, 5). On the other hand, single conidiophores with several long digitate phialides were observed on poor medium. Very sparse sporulation was a characteristic feature of the fungus. Short dense chains of conidia were produced only at a temperature of 30°C. Conidia with a smooth or slightly rough surface were cut at one or both ends (Figs. 4, 6). On Sabouraud medium, conidia were beige in mass but on other culture media, they were hyaline. Sizes on PDA were as follows: conidiophores from 50.2 to 205.0 µm, vesicles from 5.83 to 6.5 µm; phialides 8–10 × 2.4–3.2 µm and conidia 3.88–5.4 × 1.94–3.6 µm. No perithecia were found. The macro- and microscopic features of the colonies were characteristic of fungi of the genus *Aspergillus*, series *Restrictus*, and most likely *A. penicillioides* Speg. (Thom and Raper 1945; Sklenář 2017.)

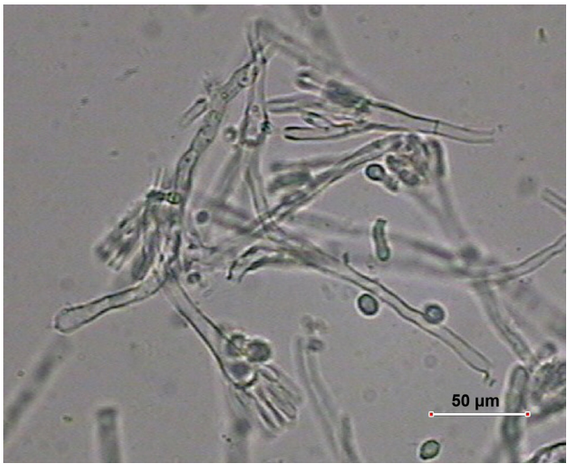


Fig. 4. Conidiophores and conidia of *A. penicillioides* on PDA.
Photo E. Zalewska.

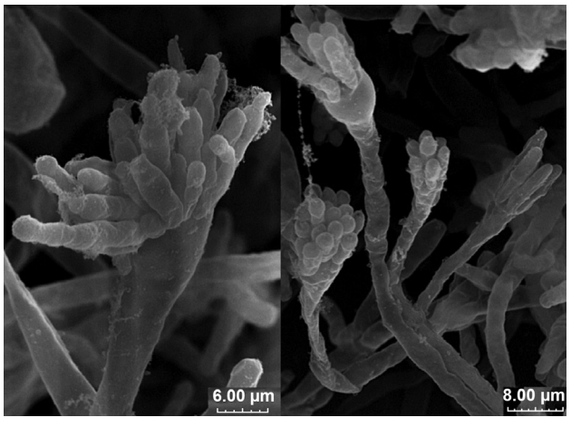


Fig. 5. Conidiophores of *A. penicillioides* on PDA.
Photo M. Wróbel.

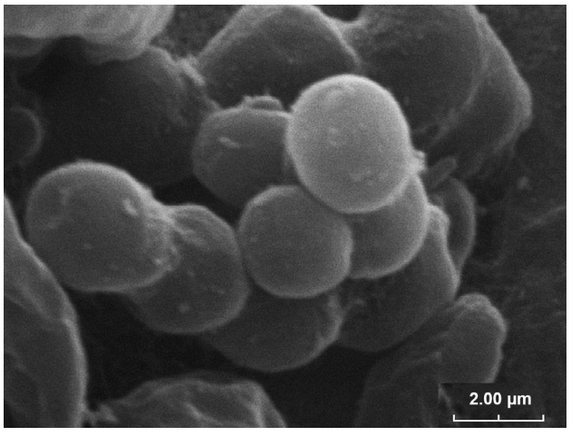


Fig. 6. Conidia of *A. penicillioides* on PDA.
Photo M. Wróbel.

**Molecular characterization.** One specific product was obtained in the PCR reaction for each studied isolate (Fig. 7). The ITS1 and ITS2 primer pair generated a product with a length of approx. 600 bp, LROR and LR5 approx. 950 bp, and $Bt_2a$ and $Bt_2b$ approx. 550 bp.

The ITS1-5.8S-ITS2 region (600 nt), a partial sequence of LSU (Large Submit RNA 25–28S, 959 nt) and part of the β-tubulin gene (550 nt) were sequenced. As a result of comparison of the sequences of ITS1-5.8S-ITS2 region, 56 sequences were selected from the GenBank database, but 43 of them (76.79%) belonged to the genus *Aspergillus*, with the degree of sequence identity ranging from 93 to 100%. Sixteen of the obtained sequences showed the highest similarity, i.e. 99–100% and seven of them belonged to the species *Aspergillus penicillioides*, five to undetermined species of the genus *Aspergillus* and four to unidentified species of fungi (Table II). The sequences of *A. penicillioides* strains: KF9864.1 (569 nt), KF373543.1 (498 nt) and *Aspergillus* sp. strain KF411572.1 (551 nt) showed 100% identity to the studied isolates. The degree of

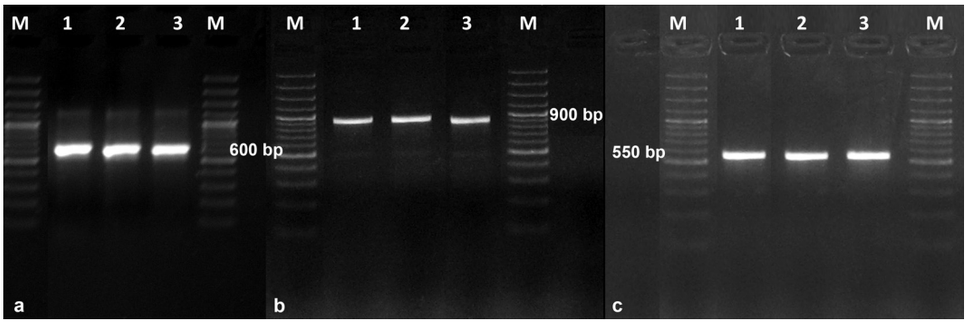


Fig. 7. Electrophoretic separation of PCR products using universal primers for regions of the genes.
a – rDNA (ITS1-5.8S-ITS4), b – LSU, c – β-tubulin. Photo A. Furmańczyk.

identity of other deposited sequences in GenBank was 99% for strains: GQ999240.1, GU17500.1, GU17494.1, FJ265955.1, FJ755819.1, KP063524.1, KP063454.1, KP974191.1 and KF22586.1 (574 nt), FR727125.1 (573 nt), FJ26597.1 (571 nt), KT959298.1 (530 nt) and for KC834810.1 (527 nt).

The comparison of 28S RNA (LSU regions) sequences of the isolates with the sequences deposited in GenBank resulted in the selection of 56 sequences. Their identity relative to the sequences tested ranged from 95 to 100% (Table II). *A. penicillioides* strain GU017535.1 was a species that showed the greatest sequence similarity (100%) over the length of about 833 nucleotides. Other three reference strains of this species showed 99% similarity; it was over 835, 791, and 783 nucleotides for the strains GU017541.1, KT390118.1 and U81265.1, respectively.

Moreover, the bioinformatic analysis of ITS1-5.8S-ITS2 and 28S RNA (LSU) regions showed a very strong similarity of our isolates to the reference strain K279 of *A. penicillioides,* the identity 99% for ITS1-5.8S-ITS2 and LSU, strain K251, ITS1-5.8S-ITS2 and LSU (100% sequence identity) (Table II). For the reference strain K279, the aligned sequences overlapped the sequences from nucleotide 6 to 590 in ITS1-5.8S-ITS2 regions, from nucleotide 1 to 871 in the 28S rRNA gene. For the strain K251 it was visible for the fragments from nucleotide 55 to 639 of ITS1-5.8S-ITS2 regions and from nucleotide 1 to 868 for the 28S rRNA gene.

The comparative analysis of the β-tubulin gene sequence showed higher variation than in the two other loci, and the identity of sequences ranged from 78 to 97%. The highest sequence identity was 97% at the same coverage percentage as the sequence deposited in NCBI. This identity was observed for 506 nucleotides and it was the sequence of CCCF 3112 strain of *Aspergillus*.

Phylogenetic analysis of ITS sequences of three isolates of *A. penicillioides* and other reference strains of *Aspergillus* indicated segregation of all isolates into two main clusters (Fig. 8). The first main cluster contained the reference strains of *A. penicillioides* and all our isolates of this species (Fig. 8). The second main cluster included the reference strains of *A. hordei* and *A. salinicola* (Fig. 8).

## Discussion

The results obtained indicated that *A. penicillioides* could be a fungus species involved in corneal inflammation. This species was described by Spegazzini et al. (1896) and it was included into *Aspergillus* series of limited growth in a monograph of Thom and Raper (1945). *A. penicillioides* firstly was isolated from mouldy sugar cane in Argentina, cured fish in Australia and desiccated chilli in New Gwinea (Spegazzini et al. 1896; Tamura et al. 2000). The first report of disseminated aspergillosis caused by *A. penicilioides* in humans was described by Gupta et al. (2015). This species was isolated during an autopsy of an infant with cystic fibrosis (Gupta et al. 2015). *A. penicillioides,* as a xerophilous species, occurs in closed spaces, dust, on dry products, clothes, mattresses and paper products (Gallo 1993; Ejdys 2007; Visagie et al. 2014; Sklenář et al. 2017). The results of aeromycological studies confirmed a very high occurrence of *Aspergillus* spp. in the air (Bayman et al. 2002; Pusz et al. 2014). It seems that the human eye is naturally exposed to *A. penicillioides*, especially in poor hygienic conditions. Conidia of thermophilic *A. penicillioides* find very advantageous thermal and humidity conditions in the eye for the development and metabolic activity (Gock et al. 2003). That is why symptoms of aspergillosis are dangerous and more frequently observed than other mycoses. The weakened immunity due to illnesses of the patient in this study could be a factor favoring colonization of the cornea, which may confirm the opportunistic nature of the parasitic fungus identified. The detailed analyses are required to identify *Aspergillus* spp. in human tissues and organs. Our results demonstrated that the isolation of *A. penicillioides* was possible, instead of extremely slow development and sporulation. The temperature

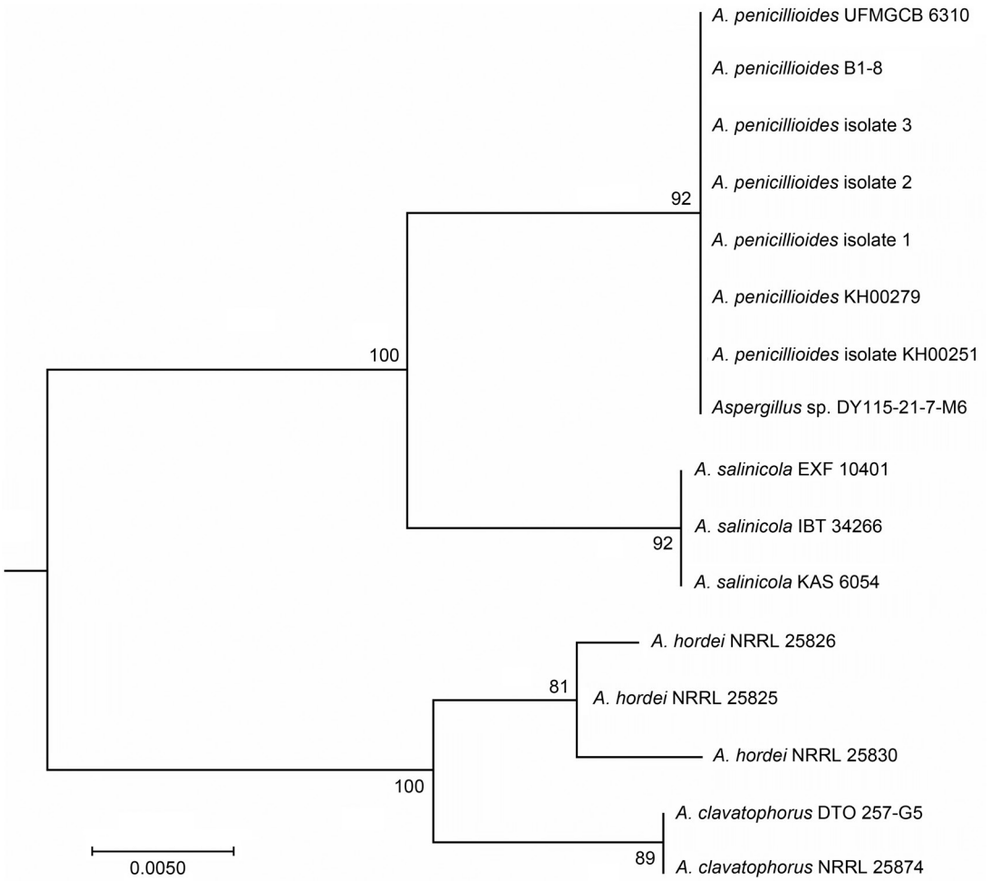


Fig. 8. Phylogenetic tree of three native *A. penicillioides* isolates and the reference strains of *Aspergillus* spp. generated using the Maximum Likelihood method.

of 25°C, suitable for the development and growth of many fugal species (Agrios 2005), appeared to be to low, regardless of culture duration. Very slow sporulation at temperature below 30°C caused serious difficulties in the culture optimization. This observation is consistent with other authors' opinion (Gock et al. 2003). The latter authors showed *A. penicillioides* sporulation at a temperature of 33°C. Among the culture media suitable for the isolation and culture of *A. penicillioides*, PDA and Sabouraud proved to be the best for the isolation and culture at a temperature of about 30°C, while above 30°C for stimulation and sporulation (Gock et al. 2003).

The presence of ambiguous microscopic features of *A. penicillioides,* for example, the overgrowth of conidiophores from aerial mycelium or substrate mycelium, various shapes and sizes of vesicle, smoothness or roughness of the surface of the conidia indicated that the identification of this species by the classical method was insufficient.

Molecular studies are required to confirm the phylogeny of *A. penicillioides.* Operons of rDNA belong to the most commonly used regions in molecular identification, as well as certain regions of mitochondrial DNA (mtDNA). Ribosomal (rDNA) regions contain both conservative regions and sequences with a very high genetic variability, such as those that separate the non-coding regions of ITS. Both are used in the diagnostics and genotyping of pathogenic fungi, and they constitute a standard method in molecular genotyping of fungi (Vilgalys and Hester 1990; Kuba 2008).

The very high degree of similarity of the studied ITS1-5.8S-ITS2 and LSU sequences indicated that the isolates analyzed belonged to *A. penicillioides*. The present study confirmed that, among the analyzed *loci*, ITS1-5.8S-ITS2 and LSU regions are useful for the identification of *A. penicillioides*.

The variable region of 28S rDNA (LSU) was detected in the genome of different species of pathogenic fungi by Sandhu et al. in 1995. The analysis of the amplified region formed the basis for the differentiation of species. Single strains of *Scopulariopsis*, i.e. *S. brevicaulis* and *S. brumptii* that cause skin and nail fungal infections were identified in this manner (Sandhu et al. 1995). However, ITS barcodes are often considered insufficient to distinguish between species (Skouboe et al. 1999; Peterson 2000, 2003; Samson et al. 2011).

Therefore, multilocus sequence analyses of the genus are used in the case of species identification as well as polygenetic analyses for *Septoria* (Quaedvlieg et al. 2013; Verkley et al. 2013), *Aspergillus*, *Penicillium* and *Talaromyces* (Visagie et al. 2014). The phylogenetic analysis of the isolates studied confirmed their identification as *A. penicillioides.*

The above results provide the first information about the colonization of the human cornea by *A. penicillioides.*

When determining the phylogeny of the studied *Aspergillus* isolates using bioinformatic methods, the strains obtained from cornea and other human tissues were not found in the available database. The compared sequences, deposited in GenBank, belonged to strains obtained from water bodies (https://www.ncbi.nlm.nih.gov/nucleotide/984294657?report=genbank&log$=nucltop&blastrank=3&RID=MSTGJ4SC01R), sea grass (https://www.ncbi.nlm.nih.gov/nucleotide/294471398?report=genbank&log$=nucltop&blast_rank=5&RID=MSFXJGHB015, microbiological materials (https://www.ncbi.nlm.nih.gov/nucleotide/221047144?report=genbank&log$=nucltop&blastrank=7&RID=MSFXJGHB015 as well as from wall paint (https://www.ncbi.nlm.nih.gov/nucleotide/951503016?report=genbank&log$=nucltop&blastrank=12&RID=MSFXJGHB015), (https://www.ncbi.nlm.nih.gov/nucleotide/927092372?report=genbank&log$=nucltop&blast_rank=15&RID=MSFXJGHB015) and personal clothing (https://www.ncbi.nlm.nih.gov/nucleotide/313574881?report=genbank&log$=nucltop&blastrank=10&RID=MSFXJGHB015.

**Conflict of interest**

Authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

# Functional and Transcriptomic Characterization of a Dye-decolorizing Fungus from *Taxus* Rhizosphere

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Abstract

We isolated three laccase-producing fungus strains from *Taxus* rhizosphere. *Myrotheium verrucaria* strain DJTU-sh7 had the highest laccase activity of 216.2 U/ml, which was increased to above 300 U/ml after optimization. DJTU-sh7 had the best decolorizing effect for three classes of reactive dyes. The DJTU-sh7-containing fungal consortium displayed the robust decolorizing ability. Both color removal efficiency and chemical oxygen demand were increased in the consortium mediated biotransformation. Transcriptome changes of *M. verrucaria* elicited by azo dye and phenolic were quantified by the high throughput transcriptome sequencing, and the activities of the selected oxidases and reductases were determined. The possible involvement of oxidases and reductases, especially laccase, aryl alcohol oxidase, and ferric reductase in the biotransformation of dye and phenolic compounds was revealed at both transcriptomic and phenotypic levels. Revealing the transcriptomic mechanisms of fungi in dealing with organic pollutants facilitates the fine-tuned manipulation of strains in developing novel bioremediation and biodegradation strategies.

Key words: *Myrothecium*, reactive dye decolorization, laccase, transcriptome sequencing, degradation mechanism

## Introduction

Nature mining for bioactive enzymes from plants and microorganisms is highlighted in building a sustainable bio-based economy. The typical reaction of laccase is the oxidation of a phenolic compound with the concurrent reduction of molecular oxygen to water (Schaechter 2009). Laccases have very broad substrate specificities and can oxidize a variety of substrates, such as di- and polyphenols, aromatic amines, and a considerable range of other compounds. Laccase is effective in dye decolorization (Bello-Gil et al. 2018; Hao et al. 2016), and it can be combined with other enzymes in the bioremediation of reactive dye (Khan and Fulekar 2017). Laccase has the potential to be implemented in various industrial fields, such as biomass conversion, waste water treatment (Jasińska et al. 2015), polymer syntheses, and remediation of bio-based chemicals, etc. Laccase-producing fungi have found uses in a wide range of technological applications (Chitradevi and Sivakumar 2011; Forootanfar and Faramarzi 2015). However, the great potential of rhizosphere as a gold ore of laccase-producing fungi has not been fully investigated and there is no report concerning about such strains from *Taxus* rhizosphere.

Currently synthetic dyes are used broadly in the textile dyeing, paper printing, color photography, pharmaceuticals, food and drink, cosmetic and leather industries. At present, more than 100 000 diverse dyes are present, with a yearly production of above 700 000 metric tons. These industries release a massive amount of colored effluents into natural water bodies, with or without treatment. The textile industry alone discharges 280 000 tons of dyes annually (Patel et al.

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2015), making it the principal contributor to tinted effluent discharge.

Although a variety of treatment technologies are accessible, e.g., adsorption, chemical oxidation, precipitation, coagulation, filtration electrolysis and photodegradation, the biological and microbiological methods using activated sludge, pure cultures, microbial consortia and degradative enzymes are cost-effective and eco-friendly alternatives. However, little information of fungi mediated decolorization is available for most dyes. For instance, the nonviable biomass of *Rhizopus nigricans* was reported to adsorb reactive green (RG) 19 (Kumari and Abraham 2007), but whether any fungus is able to degrade it is not known. Fungi efficient for azo dyes are not necessarily suitable for anthraquinone and/or phthalocyanine dyes (Baratto et al. 2015), and *vice versa*. The fungal profile for decolorizing various dye classes has not been fully investigated. Abundant data should be gathered from more extensive studies on the fungal degradation of synthetic dyes, offering researchers and industries the latest information and the expanded reference guide on the subject.

*Myrothecium verrucaria* is a species of fungus in the order Hypocreales (Sordariomycetes, Ascomycota), which is distributed globally but has not been found in the *Taxus* rhizosphere. A *M. verrucaria* strain NF-05 displays its biotechnological potential in dye decolorization (Zhao et al. 2012). *Taxus* (the source plant of the anticancer taxanes) rhizosphere can contribute to prospective biotransformation strains (Hao et al. 2008; Dou et al. 2015), which inspire us to further probe the functional potential of *Taxus* rhizosphere microbiota. We hypothesize that the *M. verrucaria* strain DJTU-sh7, with the highest laccase activity, could be effective in decolorizing three classes of reactive dyes. We also hypothesize that the transcriptomic changes of DJTU-sh7 upon azo dye elicitation could at least partially explain the underlying molecular mechanisms of fungal decolorization of azo dye. In this study, we isolated laccase-producing fungus strains, including a new *M. errucaria* strain DJTU-sh7, from the rhizosphere soil of three *Taxus* species, i.e., *T. mairei* (NF), *T. cuspidata var. nana* (ZS), and *T.* × *media* (MD). The laccase production of DJTU-sh7 was optimized via single factor experiments and the uniform design. Transcriptome analysis was performed to elucidate the molecular mechanisms of Direct Red 5B azo dye degradation in white rot fungus *Irpexlacteus* CD2 (Sun et al. 2016). RNA Seq analysis was performed to study the role of calcium chloride stress and electron transport in mitochondria for malachite green decolorization by *Aspergillus niger* (Gomaa et al. 2017). These pioneering works illustrate the power of transcriptome characterization in deciphering the fungal transformation of dye pollutants. Here, the dye removal abilities of DJTU-sh7 were characterized and the transcriptomic mechanisms of azo dye decolorization and biotransformation were revealed for the first time.

## Experimental

### Materials and Methods

**Decolorization of reactive dyes.** Six azo dyes (reactive deep blue M-2GE, reactive navy blue B-GD, reactive brilliant red KE-7B, reactive brilliant orange K-GN, RG19, and reactive black 5) were purchased from Sigma Company. Two anthraquinone dyes (reactive brilliant blue K-3R and X-BR) and the phthalocyanine dye reactive turquoise blue KN-G were from Shanghai Jiaying Chemical Engineering Co., Ltd. The degradation of nine structurally different dyes by the fungal culture/crude laccase was determined by full spectrum scan among 400–700 nm between 0–96 h. The dye decolorization was calculated at various time points. The reaction mixture for the standard assay contained respective dye (20 mg/l) in 10 mM citric acid-$Na_2HPO_4$ buffer at pH 4.5 and 1 ml of fungal suspension/crude laccase in a total volume of 10 ml. The dye decolorization (%) = $[(A_i - A_t)/A_i] \times 100$, where $A_i$ – initial absorbance of the dye, $A_t$ – absorbance of the dye along the time. All experiments were performed in triplicate. The maximal absorbance wavelength of these dyes is 612 nm (reactive deep blue), 596 nm (reactive navy blue), 565 nm (reactive brilliant red), 479 nm (reactive brilliant orange), 632 nm (RG19), 597 nm (reactive black), 623 nm (reactive brilliant blue K-3R), 596 nm (X-BR) and 601 nm (reactive turquoise blue), respectively.

One ml of RG19 at a concentration of 20–500 mg/l was mixed with 0.5 ml of each fungal suspension (two member consortium) or 0.333 ml of each fungal suspension (three member consortium) and 8 ml $H_2O$. Thus, the final concentration of RG19 was 2–50 mg/l. Each experiment was performed in triplicate at 30°C for 4 days, and the dye decolorization was determined as described above. Different reactive dyes, mixed with the equal ratio, were used in the dye decolorization. COD was determined by the standard potassium dichromate method.

**Chromatography and spectroscopy.** The degradation mixture was centrifuged, and pH of the supernatant was adjusted to 5.0. Extraction of products from the supernatant was performed using equal volume of chloroform, and the extract was then evaporated in vacuum and dried. The solid residue obtained was dissolved in 1 ml chloroform and 1 ml methanol for thin layer chromatography (TLC), and the 365 nm UV light was used to observe TLC results.

For high performance liquid chromatography (HPLC), the above solid residue was dissolved in

a small volume of HPLC-grade methanol, and the sample was then used for analytical studies. This was the test sample. A dye solution of 20 mg/l was used as a biotic control. Agilent1200 (Palo Alto, CA) HPLC conditions: C18 column, 250 × 4.6 × 5 μm, 30°C, injection volume 5 μl, flow speed 1 ml/min, mobile phase $H_2O$/acetonitrile, detection wavelength 632 nm. Gradient elution: 0–15 min, $H_2O$ 90%/acetonitrile 10%; 15–21 min, $H_2O$ 10%/acetonitrile 90%; 21–25 min, $H_2O$ 90%/acetonitrile 10%.

The biotransformation was monitored using FTIR (Fourier transform infrared) (Bio-Rad FTIR Model FTS 135) and compared with the control sample. The FTIR analysis was performed in the mid-IR region of 450–4000 $cm^{-1}$ with 16 scan speed. C18 solid phase microextraction column (Agilent) was used to extract degradation products from the filtered supernatant, with dichloromethane as the elution solvent and the elution rate 2 ml/min. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in the sample holder, and the IR scan was performed. The dye decolorization was monitored via UV-Vis spectroscopic analysis (Hitachi U-2800, Japan), using supernatants.

**RNA extraction.** The single colonies of *M. verrucaria* were inoculated into three 500 ml flasks of the following medium: sucrose 2 g, peptone 1 g, $KH_2PO_4$ 0.2 g, $MgSO_4$ 0.05 g, $CaCl_2$ 0.0075 g, $CuSO_4$ 0.001 g, $H_2O$ to 100 ml, which was control culture without guaiacol or RG19 (named "ordi"). The single colonies from the same culture plate were also inoculated into three 500 ml flasks of the ordinary medium supplemented with 0.04% guaiacol (named "guai"). Similarly, the single colony was grown in the ordinary medium supplemented with 5 mg/l RG19 (named "green"). After 5-d shaking flask culture at 25°C, the fresh medium with the same components as the initial medium of each group was added into the respective flask. The fungal mycelia of the same group were collected and mixed together for RNA extraction after another 24 h culture. Total RNA was extracted from *M. verrucaria* using the TRIzol reagent. RNA integrity was checked on a 1% agarose gel. The total RNA from three technical replicates of ordi was mixed equally, and the RNA mixture was obtained from guai and green respectively. RNA concentrations were determined using a NanoDrop 2000C spectrophotometer (Thermo Scientific, USA). RNA with OD260/280 = 1.8–2.2 and OD260/230 = 1.8 was used in the transcriptome sequencing.

**Transcriptome sequencing and bioinformatics analysis.** The transcriptome sequencing libraries of ordi, guai and green were constructed as previously described (Hao et al. 2015). The library products were sequenced via Illumina HiSeq 4000 sequencer. By base calling, the original image data for three groups (control, guaiacol and RG19 treatments) produced by the sequencer was transferred into raw reads, available at NCBI Sequence Read Archive (accession nos. SRR5738138, SRR5738139, and SRR5738140). After data cleaning, Trinity (http://trinityrnaseq.sourceforge.net/) was used in the de novo sequence assembly. Clean reads (ordi 30 334 130, guai 30 229 428, and green 29 541 586) were mapped to the assembled Unigenes, using Bowtie 2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml), for analysis of differentially expressed genes (DEGs). Unigene expression levels are presented in FPKM (Fragments Per Kb of exon per Million fragments mapped).

The functional annotations were performed by the alignments with NCBI Nt, NR, SwissProt, KOG and KEGG databases using Blast N (Nt) or Blast X/Diamond (the rest) (Buchfink et al. 2015). BLAST2GO (default parameters; http://www.blast2go.com/) and the GO database (http://www.geneontology.org) were used for the functional annotation of biological process, molecular function and cellular component. InterProScan 5 (http://www.ebi.ac.uk/interpro/search/sequence-search) was used for the InterPro annotation. Trinity and Transdecoder (https://help.rc.ufl.edu/doc/TransDecoder) were used to predict the open reading frame (ORF) and protein-coding sequence (CDS).

The software RSEM (http://deweylab.biostat.wisc.edu/rsem/) was used to calculate the gene expression level from RNA-Seq data. FDR (false discovery rate) ≤ 0.001 and the absolute value of $\log_2$fold change ≥ 1 were used as the threshold to judge the significance of gene expression difference (Hao et al. 2011). Cluster analysis of expression patterns of 21 220 Unigenes, expressed in all three conditions (ordi, guai, and green), was performed with software Mfuzz (Kumar and E Futschik 2007). The pheatmap function of R package (https://www.r-project.org/) was used to perform the hierarchical cluster analysis for DEGs.

The GO classification and KEGG pathway visualization of DEGs were performed. The pathway enrichment analysis by the phyper function of R package identified significantly enriched metabolic pathways and signal transduction pathways in DEGs compared with the reference gene background. The Q value was obtained by the Bonferroni correction of the $p$ value. Pathways with $q$ values ≤ 0.05 are significantly enriched in DEGs.

Proteins usually perform functions after they are combined into a complex by interaction. The interacting DEGs usually have similar functions. In this study, DEGs were mapped to STRING database (https://string-db.org/), and the homology between the known protein and DEG encoding protein was utilized to obtain the interaction relationship between DEG encoding proteins.

**Quantifying activities of oxidase and reductase.** The reaction mixture of 10 ml consisted of 0.04 mmol guaiacol (Sheikhi et al. 2012; Zhao et al. 2012) in 1 ml 95% ethanol, 1 ml crude enzyme, and 8 ml sodium succinate buffer, which reacted for 30 min at 30°C and were subjected to absorbance determination at 465 nm. The inactivated crude enzyme (boil for 5 min) was used as the negative control. One unit of the laccase activity was defined as the enzyme amount that was used to catalyze the oxidation of 1 nmol guaiacol within 1 min. In laccase activity (U/ml) = $10^6 \times$ reaction volume $\times \Delta A$ / (volume of crude enzyme $\times$ absorbance coefficient $\varepsilon \times \Delta t$), $\Delta A$ is the change of absorbance and $\Delta t$ is the reaction time.

Cells of pure *M. verrucaria* strains grown in culture medium containing 20 mg/l RG19 for 72 h or 96 h were collected by centrifugation (5000 × g for 20 min). The supernatant after centrifugation was used for determining the activities of extracellular enzymes. The cell pellet was washed for three times with 10 mM potassium phosphate buffer (pH 7.2) to eliminate the influence of potential extracellular enzyme on quantifying the intracellular enzyme activity. The supernatant of the ultrasonicated cell suspension was used for determining the activities of intracellular enzymes.

Enzyme activities were monitored spectrophotometrically (Shanghai Metash Instruments Co., Ltd., China) with a total volume of 10 ml at room temperature (25°C). For all enzymes, the supernatant and cell suspension were boiled for 20 min and used as the control inactivated enzyme. Laccase activity was determined as described above, except that the minimal amount of carbon and nitrogen sources were used in the liquid culture. Lignin peroxidase (LiP) reaction mixture in 50 mM sodium tartrate buffer (pH 3.0) contained 10 mM veratryl alcohol as substrate, 4 mM $H_2O_2$ and 1.0 ml enzyme. The reaction was monitored by measuring the change at 310 nm (Jasińska et al. 2015). Manganese peroxidase (MnP) reaction mixture in 50 mM sodium malonate buffer solution (pH 4.5) contained 20 mM DMP (2,6-dimethoxyphenol) as substrate, 20 mM MnSO4, 4 mM $H_2O_2$ and 1.0 ml enzyme. The reaction was monitored by measuring the change at 470 nm (Jasińska et al. 2015). Tyrosinase activity was determined by the formation of *o*-benzoquinone and dehydroascorbic acid in 10 ml reaction mixture containing 50 mM of catechol, 2.1 mM of ascorbic acid and 1.0 ml enzyme in 50 mM potassium phosphate buffer (pH 6.5) and decrease in optical density (OD) was measured at 265 nm (Waghmode et al. 2011). All enzyme assays were carried at room temperature where reference blanks that contained inactivated enzyme were run along the test. Aryl alcohol oxidase (AAO) activity was determined by the formation of aryl aldehyde in 5.0 ml reaction mixture containing 10 mM *p*-anisyl alcohol (4-methoxybenzyl alcohol) (Ferreira et al. 2005) and 1.0 ml enzyme in 100 mM phosphate buffer (pH 6.0) and OD was measured at 285 nm.

NADH-dichlorophenolindophenol (DCIP) reductase activity was determined as described by Dawkar et al. (2010). The reaction mixture in 50 mM phosphate buffer solution (pH 7.4) contained 1.72 mM DCIP (2,6-dichloroindophenol sodium salt) and 0.70 mM NADH as the substrate, and 1.0 ml enzyme. The reaction was monitored by measuring the change at 620 nm. Azo reductase activity was determined according to the method described by Ramalho et al. (2005), which monitored the change of methyl orange concentration at 461 nm. Ferric reductase activity was determined according to the method described by Ramalho et al. (2005), and the absorbance at 562 nm was measured against a blank prepared similarly but with inactivated enzyme. All determinations were performed in triplicate.

## Results and Discussion

**Fungal strains and single factor experiments.** Three laccase-producing fungal strains were isolated from the enrichment culture (Hao et al. 2016), i.e., a *Myrothecium verrucaria* (Sordariomycetes, Ascomycota) strain DJTU-sh7 from MD, a *Glomerella* (Sordariomycetes) strain from ZS, and a *Talaromyces stollii* (Eurotiomycetes, Ascomycota) strain from NF. The time course of laccase production in DJTU-sh7 was compared with those of other three laccase-producing fungi (Fig. 1). At the beginning, the mycelia grew slowly, and mycelial pellets were small with the low laccase activity in all strains. Then the mycelia grew fast and mycelial pellets became more compact and larger, while the fermentation media were clear and sticky. DJTU-sh7 displayed the highest laccase activity on day 6 (Fig. 1A), followed by the *Glomerella* strain on day 5, the *Aspergillus* strain (isolated from *Cordyceps sinensis* rhizosphere, unpublished) on day 6, and the *T. stollii* strain on day 7. The maximal mycelia dry weight came two days later than the laccase peak in DJTU-sh7 (Fig. 1B).

The process parameters that influence laccase production were optimized. The type of the carbon/nitrogen source influenced the laccase production by all strains (Fig. 1). When the carbon source was sucrose, all four strains showed the relatively higher laccase activity (Fig. 1C); when the carbon source was starch, lactose, or glucose, only DJTU-sh7 exhibited the high laccase activity; when fructose was the sole carbon source, the laccase production was meager in all strains. On the other hand, peptone could be the best nitrogen source of four strains (Fig. 1D), followed by yeast extract and beef extract, while the other four nitrogen sources were not ideal.

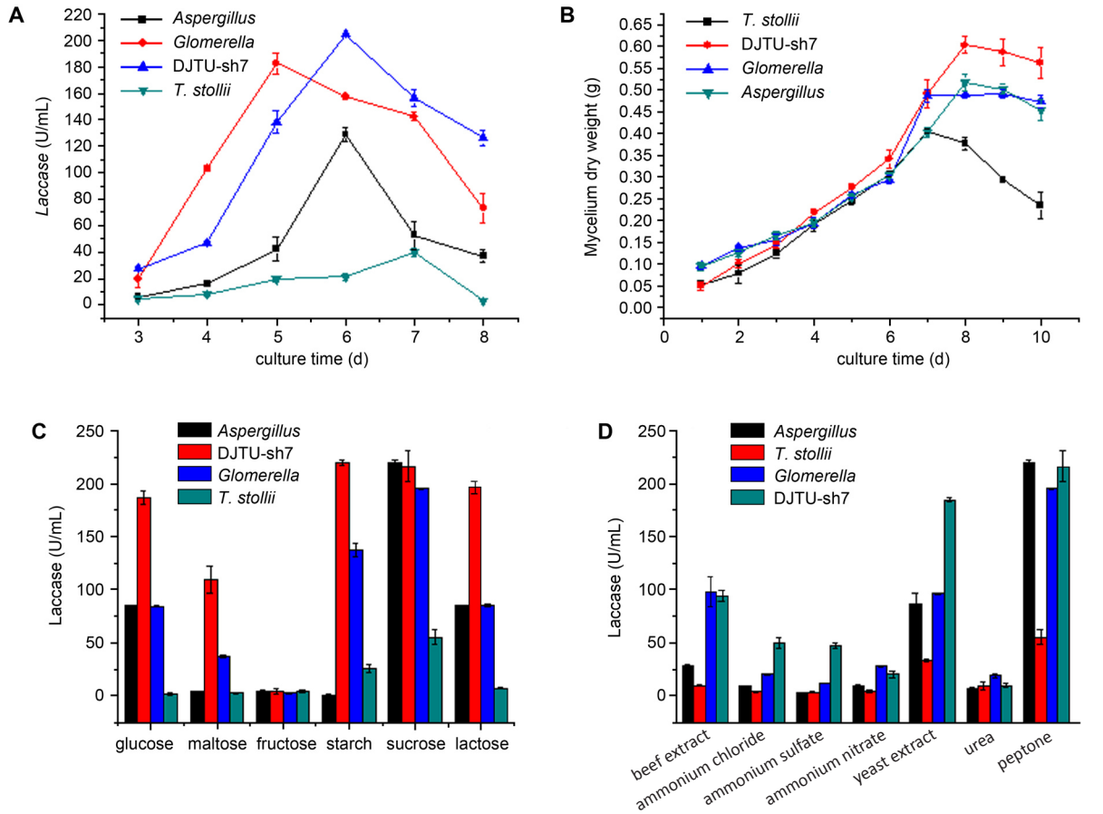


Fig. 1. A. Time course of the laccase production in four fungal strains. B. Biomass production, represented by the mycelium dry weight, of fungal strains. The 70 ml culture media contained 20 g/l glucose, 5 g/l peptone, and 5% fungal inoculant. Effects of carbon sources (C)/nitrogen sources (D) on the laccase production of four fungal strains. The agitation rate and the incubation temperature were 120 rpm and 28°C respectively.

The impacts of the incubation temperature, the initial pH, and dye concentration on the decolorization ability of DJTU-sh7 were investigated. Without fungi, adjusting pH alone did not decolorize any of the nine reactive dyes (data not shown). The maximal decolorization of 20 mg/l RG19 by the DJTU-sh7 suspension was obtained at pH 4.5 after 4 d reaction, and RG19 was decolorized within a broad range of pH 3.5–7. The maximal removal of RG19 was obtained at 35°C and pH 4.5 after 24 h shake flask culture. The conspicuous elimination of RG19 of 2–20 mg/l was observed at 35°C and pH 4.5 after 4 d reaction. The decreased decolorization of RG19 of 40–50 mg/l was obvious, implying the saturation effect of the decolorization process.

**Dye decolorization.** The whole cell biotransformation approach and the crude laccase were used to compare the dye decolorization performance of four fungal strains (Fig. S1A-I; also see Fig. 6 of Hao et al. 2016). The performance of the crude laccase (culture media and extracellular enzyme) of DJTU-sh7 was a little inferior to that of the DJTU-sh7 suspension culture (fungi, culture media and extracellular enzyme), but in most cases the difference between two approaches was less than 20%, implying that the dye biosorption of fungi and the intracellular factors were of minor role and the extracellular enzymes released from fungi played a major role in dye degradation and decolorization. Besides, the purified laccase of *M. verrucaria* NF-05 could decolorize a few other azo and anthraquinone dyes (Zhao et al. 2012).

A *M. verrucaria*-containing four-member fungal consortium was able to decolorize the azo dye cibacron yellow S-3R more efficiently than the individual fungus (Chitradevi and Sivakumar 2011). In this study, the dye-reducing fungal consortia were used to improve the decolorizing process of other dyes (Fig. 2A). The two-member consortium that consisted of DJTU-sh7 and *Glomerella* showed higher decolorizing efficiency than the individual strains at 40–50 mg/l RG19, implying the synergistic interaction between strains, while DJTU-sh7 alone was more efficient than both DJTU-sh7 and *Glomerella* at 2–10 mg/l RG19. On the contrary, *Aspergillus* and *T. stollii* was not significantly better than *Aspergillus* alone at most RG19 concentration values, and the consortium's performance at 50 mg/l substrate was much worse than that of either

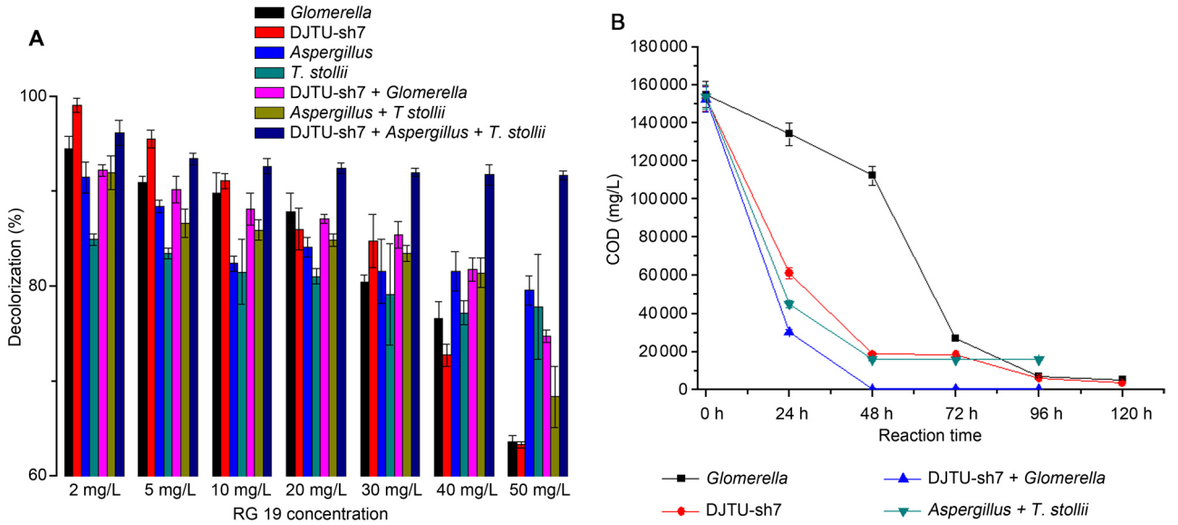


Fig. 2. A. RG19 (20 mg/l) decolorization by dye-reducing fungal consortia. B. COD removal by the fungal consortium or the single strain. The mixture of reactive dyes consists of RG19, reactive brilliant blue K-3R and X-BR (each in 6.67 mg/l). Error bars are standard deviations (n = 3).

*Aspergillus* or *T. stollii*, indicating the interspecific antagonistic interaction. Interestingly, the three-member consortium DJTU-sh7, *Aspergillus* and *T. stollii* displayed the robust decolorizing ability throughout various substrate concentrations, suggesting that these strains interact to achieve better dye decolorization. These results provide more diverse dye removal methods and selectivity, with research and development potential.

Three reactive dyes were mixed together, which simulated the industrial dye manufacturing effluent. COD was used to evaluate the mixed dye removal efficiency (Fig. 2B). Both color and COD removal efficiency was increased in the consortium mediated biotransformation, when compared with the single strain. The DJTU-sh7-containing consortium was more efficient than the one without it, and the initial COD value that could be processed by it is two orders of magnitude higher than that processed by a laccase-producing bacterial consortium for the treatment of industrial dye effluent (Patel et al. 2015).

**Spectroscopy and chromatography.** A 24-h culture of *M. verrucaria* strain DJTU-sh7 in the logarithmic phase of growth was used for the comparison studies. The UV-visible scan (400–800 nm) of medium supernatants withdrawn after 72 h of exposure to the fungi indicated decolorization and decrease in the concentration of RG19 (Fig. S2). The maximal absorbance of the decolorizing product might not fall within the range of measurements, suggesting that the dye structure was altered significantly.

The FTIR spectra before and after dye removal were compared. Peaks at 722 $cm^{-1}$ (alkene, OH bending), 1014 and 1193 $cm^{-1}$ (sulfonic acid group), and 1622 $cm^{-1}$ (C-$NH_2$) were absent after dye decolorization, suggesting the cleavage of the azo bond and the dye degradation, which was supported by the appearance of new peaks. In TLC, a few solvent systems containing 5% acetic acid were used. In all cases, an unambiguous bright blue spot, representing the decolorizing product, was observed in the organic extract of the reaction solution under UV light, while RG 19 was significantly reduced. RG19 is a naphthol type azo dye with the hydroxyl group at *ortho* to azo bond and the electron-releasing group (i.e., -NH-triazine) (Hsueh et al. 2009). The aromatic amine is commonly found in the organic extract of the azo dye decolorization (Khandare et al. 2012). Both the azo dye and its decolorizing product are highly polar and water-soluble, which was confirmed by HPLC results. The retention time of RG19 was 1.673 min, while after transformation three putative product peaks appeared at 1.338, 1.955, and 2.134 min, respectively.

**Transcriptome sequencing.** RNA from *M. Verrucaria* control samples and ones undergoing guaiacol and RG19 treatments was subjected to the Illumina HiSeq4000 paired-end sequencing, which generated 30 334 130 (ordi), 30 229 428 (guai), and 29 541 586 (green) clean reads with a total of 13.51 Gb. Trinity assembled 25 415 Unigenes (ordi 17 972, guai 21 232 and green 19 059; sequences available upon request), corresponding to 31.82 Mb. The average length of the Unigenes is 1252 bp, with N50 2333 bp and GC 54.45%. A total of 20 541 Unigenes (80.82%) have at least one annotation from one the following databases. NR, Swissprot, GO, KOG, KEGG, NT, and Interpro annotations were applicable for 75.6%, 46.4%, 42.6%, 45.6%, 51.0%,

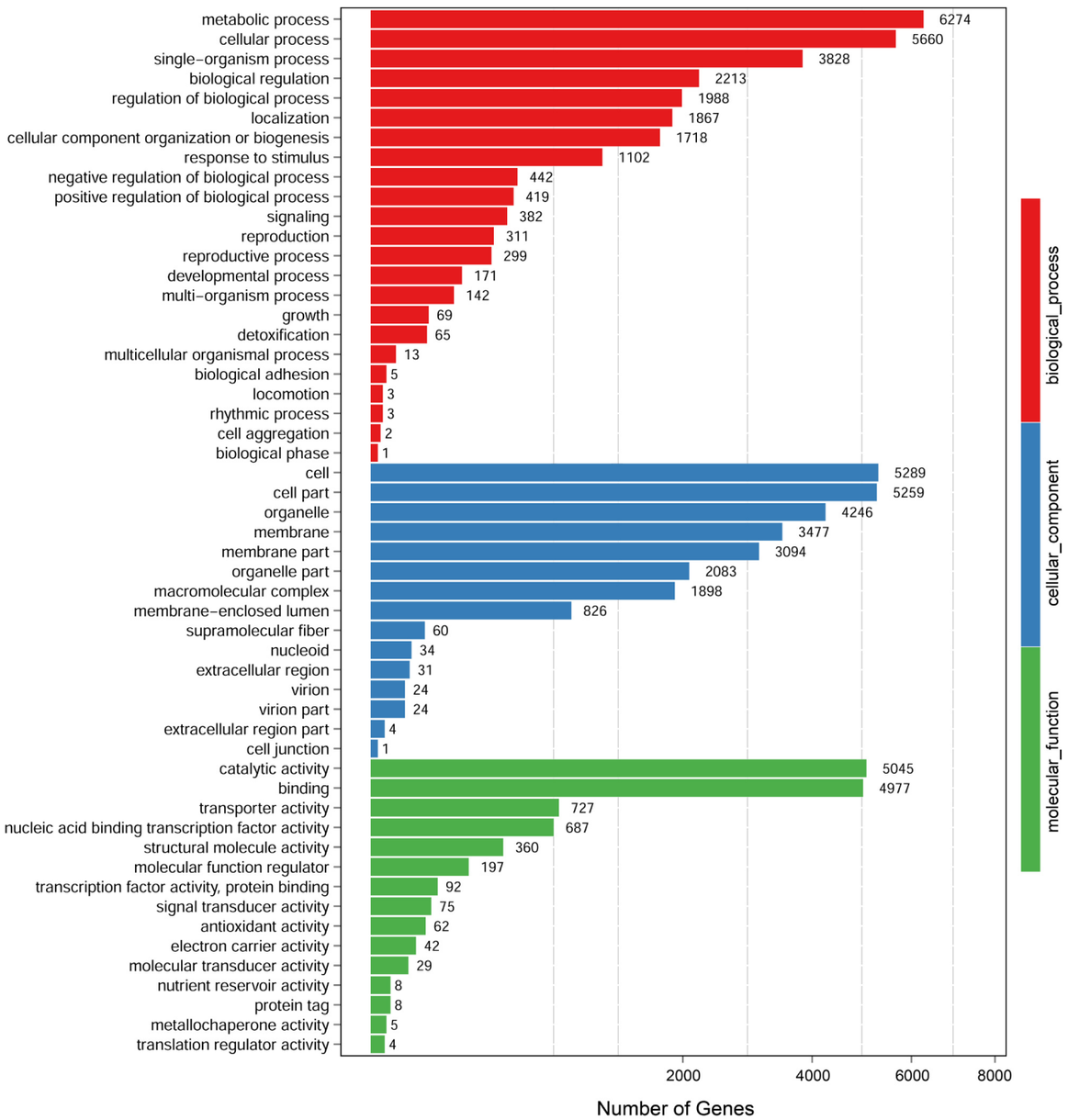


Fig. 3. A. GO annotation of biological process, cellular component and molecular function.

60.0%, and 52.7% of Unigenes, respectively. A total of 19 804 CDSs were predicted, which range between 297 and 14 538 bp, with N50 1479 bp and GC 56.0%.

In GO annotation of biological process, 6274 Unigenes belong to "metabolic process" (Fig. 3A), followed by "detoxification" (65), etc. In molecular function, 5045 Unigenes fall into "catalytic activity" (Fig. 4A), followed by "transporter activity" (727), "nucleic acid binding transcription factor (TF) activity" (687), and "antioxidant activity" (62), etc. These data indicate the great potential of *M. verrucaria* in xenobiotic metabolism and disposition. Many Unigenes involving in the biotransformation of azo dye and phenolics belong to the above GO terms. In KOG (Eukaryotic Orthologous Group) annotation, 436 and 1128 Unigenes belong to "secondary metabolite biosynthesis, transport and catabolism" and "defense mechanisms", respectively.

In KEGG annotation, 1342 (10.34% of annotated genes) belong to "Biosynthesis of secondary metabolites" and 20 belong to "Xenobiotics biodegradation and metabolism". Fifty two Unigenes belong to "Mismatch repair", followed by "Base excision repair" (52). One hundred twenty eight (0.99%) Unigenes fall into "ABC transporters". These statistics further suggest potential

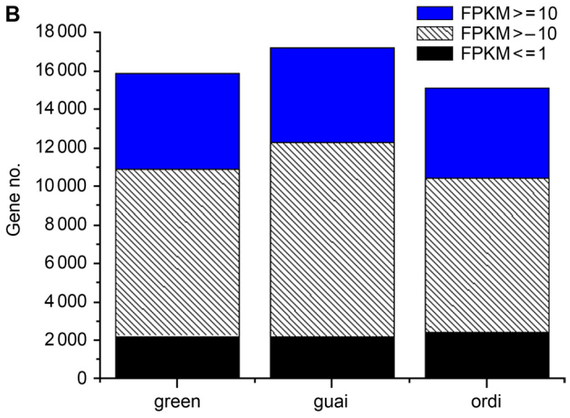


Fig. 3. B. Distribution of Unigene expression levels.

of our *Myrothecium* strain in azo dye and phenolic biotransformation.

In the control sample, 89.3% of clean reads were mapped onto the de novo-assembled Unigenes, while 88.2% and 86.6% of reads were mapped to the Unigenes in guai and green, respectively. In total, 15 123, 17 171, and 15 884 Unigenes were expressed in ordi, guai, and green, respectively, indicating that organic compound treatments induced extensive elicitor-specific transcriptome remodeling. The most abundant Unigenes in the three conditions were distinct. These results indicate dramatic transcriptome remodeling and reprogramming in *M. verrucaria* following azo dye treatment. According to the Unigene expression level distribution (Fig. 3B), in three conditions more Unigenes had the expression level between FPKM 1 and 10, followed by FPKM ≥ 10 and ≤ 1. The overlapping peaks of three conditions around $\log_{10}$FPKM 0.5 suggest that 5 mg/l RG19 was not highly toxic to the fungal strain.

Cluster analysis of expression patterns of 21 220 shared Unigenes among all three conditions (ordi, guai, and green) was performed with software Mfuzz. These genes were divided into 12 clusters. In clusters 3 (1310 Unigenes), 5 (2988), 8 (1042) and 12 (1240), the gene expression level in green was higher than those in ordi and guai. Most differentially expressed genes (DEGs) involved in azo dye biotransformation and degradation are included in these clusters and are highlighted in the following analyses.

**DEGs.** A total of 3586, 2159 and 3796 Unigenes had statistically significant expression differences between ordi and guai, ordi and green, and guai and green, respectively (Fig. 3C). In total 623, 445 and 674 Unigenes showed significantly different expression only between ordi and guai, ordi and green, and guai and green, respectively (Fig. 3D), but not in other two comparisons. For the DEGs, $\log_2$FC of ordi expression vs. guai expression ranged from –12.0 to 14.5. $\text{Log}_2$FC values of ordi vs. green and guai vs. green varied from – 11.0 to 11.7 and –14.5 to 12.7, respectively. It seems that 0.04% guaiacol elicited stronger and more

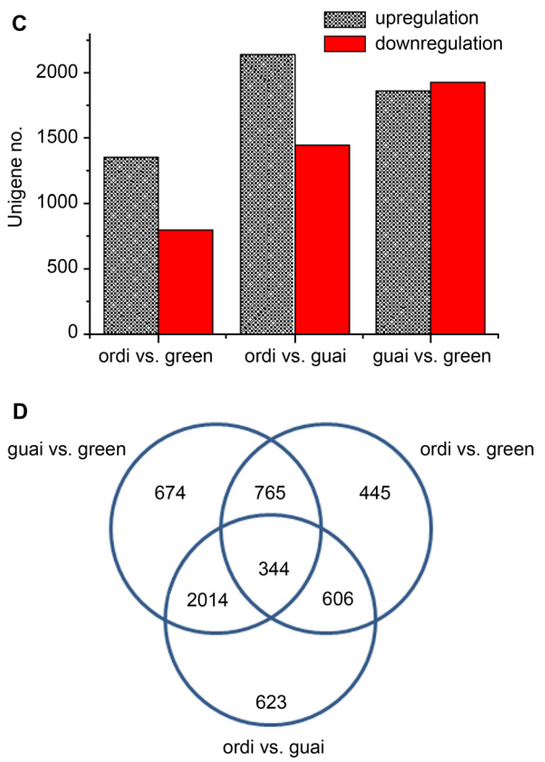


Fig. 3. C. No. of DEGs in three comparisons. In a pairwise comparison, the former one is the control and the latter the treatment. D. Common and unique DEGs of three comparisons.

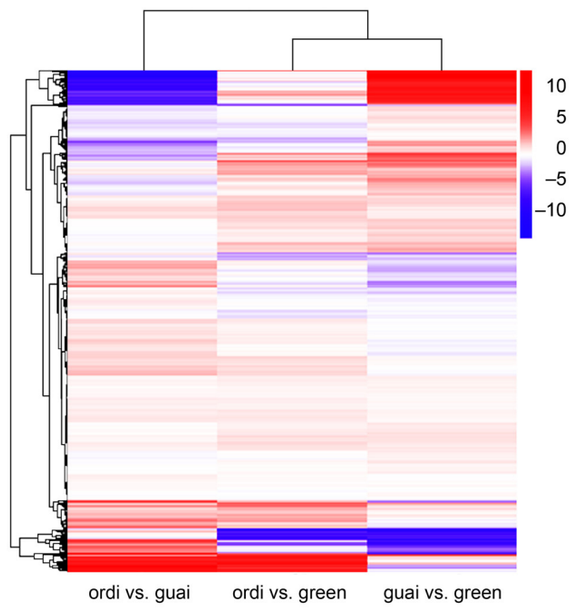


Fig. 4. Hierarchical cluster analysis of DEG union set of ordi vs. green, ordi vs. guai, and guai vs. green. The redder the color, the greater the upregulation of the gene expression; the bluer the color, the greater the downregulation of the gene expression.

extensive responses in the gene expression of *M. verrucaria* than 5 mg/l RG19.

Genes with similar expression patterns usually have the functional correlation. In the cluster analysis, 9795 DEGs fall into scores of unique clusters showing distinct expression patterns in the union set of ordi vs. guai, ordi vs. green, and guai vs. green (Fig. 4). Some clusters were identified, in which most Unigenes were upregulated in green when compared with ordi, and either upregulated, downregulated, or unaltered in guai as compared with ordi. These clusters might contain genes closely associated with dye biotransformation and decolorization. Many genes in these clusters are also involved in stress and defense responses.

The initial step of azo dye metabolism in many filamentous fungi involves the action of laccase (multicopper oxidase) and peroxidases (Fig. 5) (Solis et al. 2012; Jasińska et al. 2015). Iron transport multicopper oxidase fio1 (Unigene4094_All) was included in an 86-gene cluster and was significantly upregulated in green as compared with guai and ordi. Catalase and peroxidases are evolutionarily closely related. At least five Unigenes, representing catalase/peroxidase, had significantly higher expression in green than in other two conditions, as an essential response to oxidative stress triggered by RG19.

Monooxygenases, such as cytochrome p450 (CYP) and flavin-containing monooxygenase (FMO) are well known phase I xenobiotic metabolizing enzymes (Hao and Xiao 2011; Ma et al. 2017). At least 22 monooxygenase genes, e.g., benzoate 4-monooxygenase (five Unigenes), phenol 2-monooxygenase (four Unigenes), and dimethylaniline monooxygenase (three Unigenes), were upregulated significantly in green. For FAD-dependent monooxygenase (Unigene3165_All), the $\log_2$FC values in ordi vs. guai, ordi vs. green, and guai vs. green were

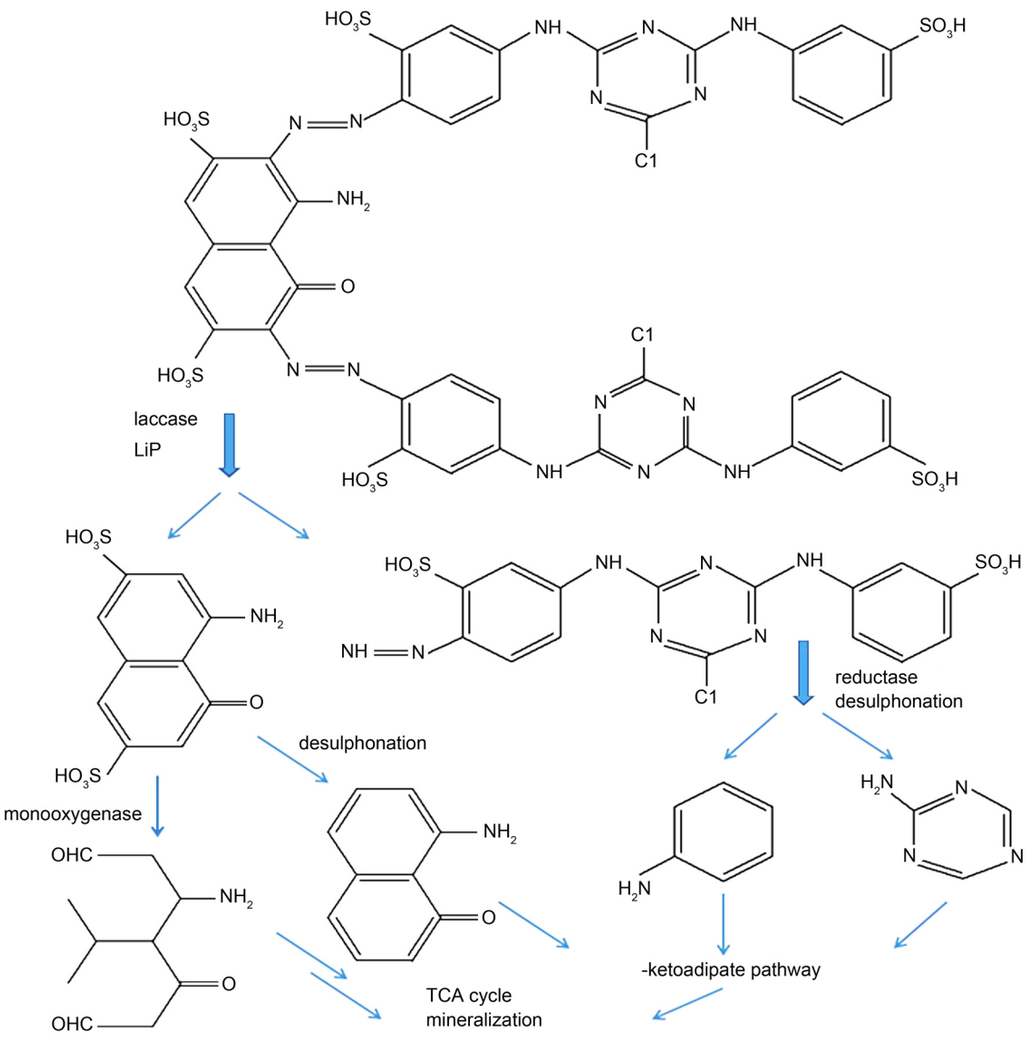


Fig. 5. Proposed degradation pathway of RG19 in *M. verrucaria*.

1.68, 3.05, and 1.38 respectively; for FMO (dimethylaniline monooxygenase CL1649.Contig2_All), the corresponding values were –0.93, 1.15, and 2.08 respectively. Alkane sulfonate monooxygenase (EC 1.14.14.5; Unigene302_All) catalyzes the transformation of alkane sulfonate ($R\text{-}CH_2\text{-}SO_3H$) + $FMNH_2$ + $O_2$ into aldehyde (R-CHO) + FMN + sulfite + $H_2O$ (Zhan et al. 2008), and thus might be indispensable in azo dye desulfonation. The $\log_2$FC values of this gene in ordi vs. guai, ordi vs. green, and guai vs. green were 0.69, 1.16, and 0.47 respectively.

Phenylacetate 2-hydroxylase catalyzes the first step of phenylacetate catabolism (Rodríguez-Sáiz et al. 2001), which generates fumarate and acetoacetate and bridges between azo dye degradation intermediate and TCA cycle. Five Unigenes of this CYP were significantly increased in green. Salicylate hydroxylase [EC:1.14.13.1], catalyzing the transformation of salicylate + NADH + $2H^+$ + $O_2$ into catechol + $NAD^+$ + $H_2O$ + $CO_2$, might be essential in the complete degradation of both azo dye and phenolic, as three Unigenes of this monooxygenase were significantly upregulated in both green and guai. Epoxide hydrolase (Unigene8943_All), exerting its function following CYP, was upregulated only in green. These enzymes might participate in the conversion of azo dye degradation intermediates.

Induction of the activity of aerobic enzyme dioxygenase indicates its involvement in dye removal (Cirik et al. 2013; Nor et al. 2015). Aromatic-ring-hydroxylating dioxygenases (ARHD) incorporate $O_2$ into their substrates in the dihydroxylation reaction. The product is (substituted) cis-1,2-dihydroxycyclohexadiene, which is subsequently converted to (substituted) benzene glycol by a cis-diol dehydrogenase, followed by ring opening. The substantially increased expression of ARHD (Unigene3184), dioxygenases (CL864.Contig1_All and Unigene3045), and isomerase (Unigene2274) suggests their prominent role in the *Myrothecium* degradation of azo dye. Our transcriptome evidence support that *Myrothecium* is able to use the β-ketoadipate pathway in degrading dye related aromatic compounds (Kim et al. 2007).

Except CYP and peroxidase, other enzymes involved in the oxidative reactions were also increased at the mRNA level. For example, tyrosinase-encoding Unigene2982_All was significantly upregulated in green ($\log_2$FC 1.81 in ordi vs. green) rather than in guai ($\log_2$FC 0.6 in ordi vs. guai) or ordi. AAO encoding Unigene7311_All had much more mRNA expression in green ($\log_2$FC 6.08 in ordi vs. green) than in guai ($\log_2$FC –1.51 in ordi vs. guai) or ordi. This enzyme was also involved in the decolorization of azo dye in *Pleurotus eryngii* (Akpinar and Urek 2014) and *Thanatephorus cucumeris* (Shimokawa et al. 2008). In contrast, the expression of MnP encoding Unigene6659_All was highest in ordi (FPKM 12.36), followed by green (6.36) and guai (2.76). Aromatic amines are metabolic intermediates during azo dye degradation (Sun et al. 2017). Copper amine oxidases (CAOs) are responsible for the oxidative deamination of aromatic amines to their corresponding aldehydes (Klema et al. 2013). The expression of CAO encoding Unigene2105_All was significantly increased in guai (FPKM 11.36) and green (1.82) as compared with ordi (0.27).

The role of reductase in the dye decolorization cannot be neglected (Song et al. 2017), especially when the oxygen supply is not sufficient. Ferric reductase-encoding Unigene558_All and Unigene7872_All were dramatically upregulated in green ($\log_2$FC1.79 and 1.2 respectively in ordi vs. green) instead of guai. In two bacterial strains, supplementation of 5 mM ferric chloride increased azo dye decolorization rate (Ng et al. 2014); ferric reductase activity was consistent with synergistic effects of ferric chloride and ferric citrate on these strains. Ferric reductase is membrane associated and involved in respiratory electron transport (Xu et al. 2007). Azo reduction by bacterial strains is coupled to the oxidation of electron donors and linked to the electron transport and energy conservation in the cell membrane (Hong and Gu 2010). Our transcriptomic and phenotypic (see below) results represent the first example of the role of ferric reductase in fungal degradation of azo dye. On the other hand, the enhanced cellular oxidation against the azo dye attack could be balanced by the upregulation of ferric reductase, which might protect cell wall integrity and mitochondrial function (Yu et al. 2014), and improve the oxidative stress tolerance (Xu et al. 2014). Correspondingly, manganese superoxide dismutase (SOD; Unigene3085_All) and SOD[Cu-Zn] (Unigene6367_All) were upregulated in green to attenuate the deleterious effect of reactive oxygen species (ROS). Unigene4933_All, representing glutathione S-transferase (GST), was upregulated to exert antioxidant activities. Both GST and N-acetyltransferase (NAT) are phase II xenobiotic metabolizing enzymes (Hao et al. 2010). NAT enables acetyl coenzyme A-dependent detoxification of aromatic amines (Cocaign et al. 2013). Five Unigenes, representing NATs, were significantly upregulated in green as compared with ordi, one of which (Unigene622_All) clustered with laccase.

**KEGG pathway enrichment analyses of DEGs.** Significantly enriched metabolic pathways of DEGs after RG19 treatment were identified. After Bonferroni corrections of *p* values, eight, 14, and 24 KEGG pathways are still enriched in DEGs of ordi vs. green, ordi vs. guai and guai vs. green, respectively. "Biosynthesis of secondary metabolites" and "Metabolic pathways" were significantly enriched in all three comparisons (Fig. S3),

indicating the enhancement of secondary metabolite biosynthesis and the overall metabolism in response to the attack of azo dye/phenolic. In guai vs. green, the enrichment of "Ascorbate and aldarate metabolism" and "Nitrogen metabolism" suggests the increased antioxidant activity and enhanced nitrogen turnover during azo dye biodegradation and metabolism, respectively; "Carbon metabolism" and "MAPK signaling pathway" were also more active after azo dye stimulation. The changes in "Biosynthesis of antibiotics" are implicated as defense responses.

**Enzyme activity.** Transcriptome sequencing results suggest that both enzymes: oxidase, e.g., laccase, AAO, tyrosinase, peroxidase, and reductase including ferric reductase could be involved in biodegradation of azo dyes. Azo reductase and NADH-DCIP reductase were involved in reductive cleavage of azo bonds, which was the first step of biodegradation of azo compounds in some microbes (Solis et al. 2012; Song et al. 2017). Although the gene expression of these two reductases was not found to be upregulated in the transcriptome analysis, their activities, along with those of other six enzymes, were quantified. The activity of MnP was not detected intracellularly and extracellularly, which corresponded to the significant downregulation of MnP expression after RG19 treatment. On the contrary, LiP can be quantified intracellularly and extracellularly (Table I), and its activity was higher in the presence of RG19 when compared with no RG19, albeit the differences between two conditions were not statistically significant. Tyrosinase also showed the trend of higher activity in the presence of RG19 when compared with no RG19, regardless of intracellularly or extracellularly, but the differences between dye presence and dye absence were not statistically significant. In contrast, RG19 significantly induced the extracellular laccase activity (Table I); RG19 also substantially induced the intracellular and extracellular AAO activity. These data, taken together with transcriptome results, suggest the major role of laccase and AAO, and minor role of LiP and tyrosinase in decolorization and biotransformation of azo dye in *M. verrucaria* strain DJTU-sh7.

NADH-DCIP reductase was involved in cleavage of azo bonds (Song et al. 2017), which were the chromophoric groups of azo dyes. NADH-DCIP reductase was detected intracellularly and extracellularly in the presence or absence of RG19, and the extracellular enzyme activity was higher than the intracellular one. The differences of NADH-DCIP reductase activity were not statistically significant between RG19 treatment and no RG19 (Table I), so were the differences of azo reductase. These data suggest that the activity of both reductases was not induced by RG19. Rather, the activity of ferric reductase might be induced by RG19, as its intracellular and extracellular activity was significantly higher after RG19 exposure. This suggests its prominent role in decolorization and biotransformation of azo dye in *M. verrucaria*.

## Conclusion and prospect

For the first time, laccase-producing fungi were isolated from *Taxus* rhizosphere. The statistically-based uniform design is well suited for optimizing the laccase-producing medium composition. *M. verrucaria* strain DJTU-sh7 proved to be most efficient in decolorizing nine reactive dyes at a broad range of pH and temperature, and the dye degradation was confirmed by chromatographic and spectral analyses. The good performance of DJTU-sh7-containing fungal consortium implies its utility in the treatment of industrial dye effluent.

The characterization of *M. verrucaria* DJTU-sh7 was strengthened by the mechanism views contributed by the transcriptome characterization of this dye-decolorizing strain. The gene expressions of multiple oxidases and reductases were significantly upregulated after azo

Table I
Enzyme activities of *M. verrucaria* strain DJTU-sh7 in decolorizing RG19.

| Enzyme (U/ml) | RG19 addition | | No RG19 | |
|---|---|---|---|---|
| | Intracellular | Extracellular | Intracellular | Extracellular |
| Laccase | ND | 26.241 ± 0.760* | ND | 17.108 ± 0.895 |
| Lignin peroxidase | 0.164 ± 0.006^ | 0.120 ± 0.006^ | 0.152 ± 0.017 | 0.097 ± 0.002 |
| Mn peroxidase | ND | ND | ND | ND |
| Tyrosinase | 45.000 ± 3.000^ | 38.667 ± 8.386^ | 42.333 ± 6.429 | 33.667 ± 2.309 |
| Aryl alcohol oxidase | 14.093 ± 0.056* | 5.900 ± 0.001* | 5.244 ± 0.056 | 3.572 ± 0.056 |
| Ferric reductase | 7.845 ± 0.137** | 11.788 ± 0.182* | 6.849 ± 0.068 | 9.000 ± 0.182 |
| Azo reductase | 22.853 ± 0.544^ | 12.696 ± 0.670^ | 23.242 ± 0.155 | 12.204 ± 0.432 |
| NADH-DCIP reductase | 3.644 ± 1.547^ | 8.926 ± 0.696^ | 2.603 ± 1.610 | 7.550 ± 2.260 |

Values are mean ± SD; *$p < 0.001$, **$p < 0.01$, ^$p > 0.05$ in Student's t-test as compared with no RG19. ND, not detected.

dye exposure, and the metabolic pathways underwent dramatic remodeling and reprogramming. Enzyme activity quantification highlighted the potentially important roles of laccase, aryl alcohol oxidase, and ferric reductase in decolorization and degradation of azo dye, which ultimately lead to mineralization through TCA cycle. Although extracellular laccase might be a major factor in the decolorization of the studied dyes, genes implicated in transcriptome analysis of dye transformation call for further mechanism studies and the process optimization at the industrial scale.

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**Conflict of interest**

Authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

# A Novel Approach to Study the Effect of Ciprofloxacin on Biofilms of *Corynebacterium* spp. Using Confocal Laser Scanning Microscopy

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Abstract

Non-diphtherial corynebacteria are Gram-positive rods that cause opportunistic infections, what is supported by their ability to produce biofilm on artificial surfaces. In this study, the characteristic of the biofilm produced on vascular and urological catheters was determined using a confocal microscopy for the most frequently involved in infections diphtheroid species. They were represented by the reference strains of *Corynebacterium striatum* ATCC 6940 and *C. amycolatum* ATCC 700207. The effect of ciprofloxacin on the biofilm produced by the antibiotic-susceptible *C. striatum* strain was evaluated using three concentrations of the antimicrobial agent (2×, 4×, and 6× the MIC – the Minimum Inhibitory Concentration). The basis for the interpretation of results was the statistical analysis of maximum points readings from the surface comprising a total of 245 areas of the biofilm image under the confocal microscope. It was observed that ciprofloxacin at a concentration equal to 4×MIC paradoxically caused an enlargement of areas with live bacteria within the biofilm. Biofilm destruction required the application of ciprofloxacin at a concentration higher than 6×MIC. This suggests that the use of relatively low doses of antimicrobial agents may increase the number of live bacteria within the biofilm, and further facilitate their detachment from the biofilm's structure thus leading to the spread of bacteria into the bloodstream or to the neighboring tissues. The method of biofilm analysis presented here provides the original and novel approach to the investigation of the diphtheroid biofilms and their interaction with antimicrobial agents.

Key words: *Corynebacterium amycolatum, Corynebacterium striatum,* biofilm, ciprofloxacin, confocal microscopy

## Introduction

*Corynebacterium striatum* is a member of microbiota of the human skin and mucous membranes but it can also cause opportunistic infections. These infections typically involve immunocompromised patients and are associated with circumstances favoring bacterial translocation from the mucous membranes or the skin through catheters and intubation or resulting from treatment that lead to disruption of tissue integrity. An important feature of *Corynebacterium* species, which promotes infections, is their ability to produce a biofilm on artificial surfaces (Mattos-Guaraldi and Formiga 1991; Kwaszewska et al. 2006; Sousa et al. 2011; Gomes et al. 2013; de Souza et al. 2015; Leal et al. 2016, Qin et al. 2017; Kang et al. 2018). When it occurs, the intravenous biomaterial's surface colonization may lead to a bloodstream infection (Martínez-Martínez et al. 1995; Moore and Norton 1995; Campanile et al. 2009), urinary tract infections (Soriano et al. 2009), infections associated with the implant surface (Merhej et al. 2009) or as the result of intubation can cause respiratory tract infections (Williams et al. 2012; Wojewoda et al. 2012). A subacute and slow process of infection often causes diagnostic problems. Antimicrobial agents are administered, but due to the occurrence of multidrug-resistant strains (Campanile et al. 2009) these infections may cause serious therapeutic problems. Antimicrobials do not destroy bacterial biofilm when applied at a concentration equal MIC and subMICs (Bridier et al. 2011; Gomes et al. 2013).

Revealing the effect of antimicrobial agents on biofilms produced by opportunistic *Corynebacterium* species is particularly important because of poorly understood process of biofilm formation by this group of bacteria and the effect of antimicrobial agents on this bacterial structure. The reference strains of *C. striatum* ATCC 6940 and *C. amycolatum* ATCC 700207 were

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used in the study to investigate their ability to produce biofilm on catheters' surface. *C. striatum* ATCC6940, which is susceptible to all antimicrobial agents, was used to analyze the effects of ciprofloxacin on the resultant biofilm structure. This antimicrobial agent inhibits bacterial DNA synthesis through its effect on the A subunit of gyrase and is used as one of options for treatment of infections caused by these bacteria.

## Experimental

### Materials and Methods

**The in vitro biofilm formation on intravenous, urinary catheter, and glass surface.** The inoculum of 0.5 McFarland turbidity was prepared using a 24-hour culture of *C. striatum* ATCC 6940 and *C. amycolatum* ATCC 700207 strains grown on the Columbia Agar medium with 5% sheep blood (bioMérieux), and was subsequently diluted 1:100 with the TSB (Tryptase-Soy Broth, bioMérieux) supplemented with 5% FCS (Fetal Calf Serum, Sigma). The approximately 1 cm fragments, cut lengthwise from vascular (Exeflon) and urological (silicon, RÜSCH) catheters were placed in the medium with bacteria prepared in the LAB-TEK Chamber Slide SYSTEM (Nunc) containers and the incubation was carried out for 48 hours at 37°C under aerobic conditions. After this period, the liquid medium was removed and the biofilm was gently washed with the PBS (Phosphate-Buffered Saline) solution to remove free-floating bacteria. In the next step, SYTO-13 (Sigma) was used for the biofilm staining according to the manufacturer's instructions (1 μg SYTO-13 per 1 ml of the PIPES buffer for 15 minutes at room temperature). In a similar manner, development of the biofilm on the surface of glass (glass coverslip placed in the LAB-TEK Chamber Slide System container) for *C. amycolatum* and *C. striatum* was tested. The biofilm surface was analyzed with a confocal microscope – LSM 5 PASCAL (Zeiss), with 50× magnification, and the images were taken and archived.

**The effect of ciprofloxacin on the *C. striatum* ATCC 6940 biofilm produced *in vitro*.** The reference strain *C. striatum* ATCC 6940 is susceptible to antimicrobial agents including ciprofloxacin (MIC = 0.125 μg/l). The biofilm was developed on the surface of the LAB-TEK Chaber Sidle System (Nunc) in the TSB with 5% FCS, using an inoculum of 0.5 McFarland turbidity, diluted in the medium (1:100). The incubation was carried out for 48 hours at 37°C under aerobic conditions. After the incubation period, solutions of ciprofloxacin (Ciprofloxacin, Polfa, Poland) were added in three increasing concentrations: MIC2 = 2 × MIC (i.e., 0.25 μg/l); MIC4 = 4 × MIC (0.5 μg/l); MIC6 = 6 × MIC (0.75 μg/l) to three 48-hour independent cultures carried out parallelly in the same conditions. The assays were made in duplicate simultaneously and in the same conditions. We used the preparation of ciprofloxacin used for injections in the treatment of infections. The incubation of the biofilm with the antibiotic solutions was carried out for 2 hours in similar conditions as for the previous biofilms culture. In parallel, control cultures were carried out without the antimicrobial agent. After incubation, the liquid medium was gently removed from over the biofilm, PBS was used for washing and the surface was stained with two dyes (SYTO-13 and propidium iodide).

In order to distinguish live from dead cells within the biofilm, the staining with two dyes was performed. The SYTO-13 staining (1 μg SYTO-13 per 1 ml PIPES buffer for 15 minutes at room temperature) enabled the detection of live cells (live, green fluorescence), whereas propidium iodine (20 μg/ml) enabled the detection of damaged or dead cells, with compromised membranes (dead, red fluorescence). Images were taken using a microscope Axiovert 200M coupled with a camera (magnification 200 times).

**The evaluation of ciprofloxacin effect on the growth of *C. striatum* ATCC 6940 biofilm based on the confocal microscope image analysis.** The following biofilm parameters were determined: an area occupied by the biofilm (live and dead bacteria), the intensity of biofilm luminosity (live and dead bacteria) and the value of the maxima of luminosity within the space occupied by the biofilm (live and dead bacteria). The measurements were analyzed against the control culture without the antibiotic. The resulting images (in 8-bit grayscale) were analyzed using the program ImageJ v.1.40g, Wayne Rasband, National Institutes of Health, USA.

**Interpretation of the biofilm morphology measurements with the quantity of maxima.** The algorithm "Find Maxima" allows for determination of the number of sites in the image that differ from the surrounding areas by a given value (in 8-bit grayscale, where 0 = black and 255 = white). In case of a homogeneous biofilm, the number of points differing from the environment is lower than for the heterogeneous biofilm. Due to the fact that in the same image of biofilm, depending on the setting – "the threshold of the difference between the object and the background" may lead to different results, the number of sites differing from the background and from the minimum value of 10 to 255 were analyzed in order to reduce the error that results from the arbitrarily set values, which allow distinguishing between objects and the background. The number of counted maxima readings allowed for an objective assessment of the biofilm parameters, repeatable in all tests.

The analysis included the biofilm surface, which consisted of 245 scanned parts of the image. It covered

the whole analyzed surface of the biofilm observed in the microscope (ca. 1 $cm^2$) under the influence of each of the multiple MIC values. This allowed for the presentation of the results from maxima readings as a statistical unit. At the same time the uneven formation of biofilm structures on the analyzed surface could be taken into account, which was visible in the "scatter" of the resulting values, and which finally allowed for the statistical evaluation and comparison of effects of various doses of ciprofloxacin on the entire biofilm surface.

**The statistical analysis.** The statistical analysis of the results was performed using the Statistica 9.0 GB. The compliance of distribution of individual variables within groups with the normal distribution was verified using the Kolmogorov-Smirnov test with application of the Lilliefors' test and the Shapiro-Wilk test. As the distribution of the tested variables significantly differed from the normal distribution, nonparametric tests were used in the further analysis. For comparison of the differences between the tested groups, the Kruskal-Wallis test was used. The results were accepted as statistically significant at the significance level $p \leq 0.05$ and presented as the median (Me), and the average value (x), the minimum value (min) and maximum value (max) of the statistical series.

## Results

**The biofilm on the surface of the vascular, urinary catheters and glass surface.** The biofilm produced by *C. striatum* ATCC 6940 (Fig. 1A) on the surface of the vascular catheter (exaflon) was shown as multiple aggregates, with a small area, yet reaching in the central part of the resulting structure the value of 25 µm. The structures in the range of values from 10 to 15 µm prevailed. They were compact with a homogeneous bacterial mass, which might be observed in the cross-section (Fig. 2).

*C. amycolatum* ATCC 700207 (Fig. 1B) produced the biofilm with a larger area bit still relatively small, with a visible tangled structure, and the peak occupied a small part with a height above 15 µm.

The biofilm produced by *C. striatum* ATCC 6940 (Fig. 3A) on the surface of the urinary silicone catheter formed a large flat surface of the height up to 9 µm. A more diversified structure of a very large area was produced by *C. amycolatum* ATCC 700207 (Fig. 3B). It looked like large granular aggregates reaching the height of up to 35 µm.

The cross-section (Fig. 4) showed the diversified fiber structure with numerous channels, separated by the areas of compact bacterial mass.

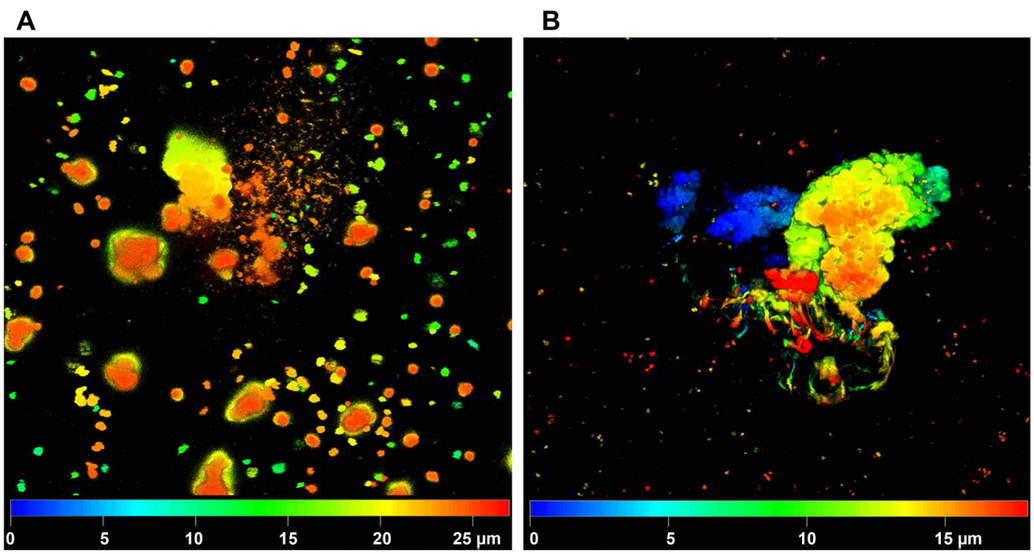


Fig. 1. The biofilms on the surface of the vascular catheter after 48 hours of incubation, stained with SYTO-13 with a marked height of the visible structures, A – *C. striatum* ATCC 6940, B – *C. amycolatum* ATCC 700207 (above 50×).

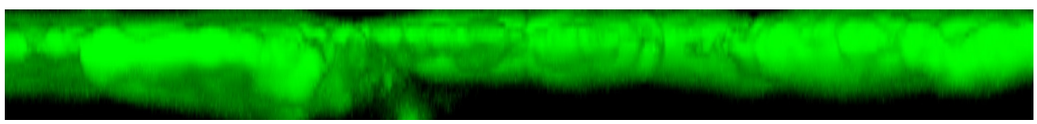

Fig. 2. The cross-section of the biofilm produced on the surface of the vascular catheter after 48 hours, stained with SYTO-13 – *C. striatum* ATCC 6940 (above 50×).

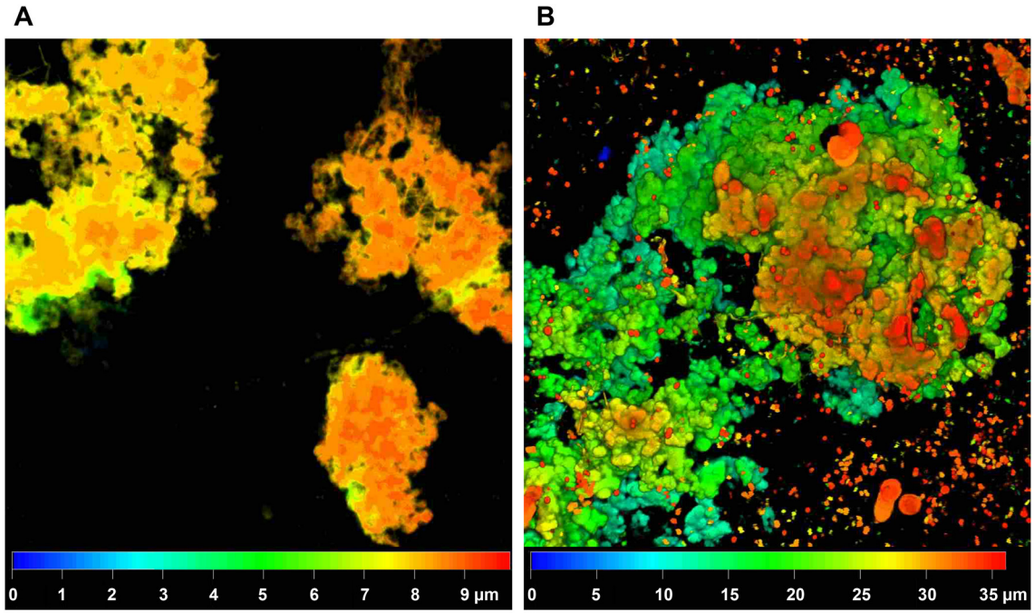


Fig. 3. The biofilm produced on the surface of the urological catheter after 48 hours of incubation, stained with SYTO-13 with a marked height of the visible structures (above 50×), A – *C. striatum* ATCC 6940, B – *C. amycolatum* ATCC 700207.

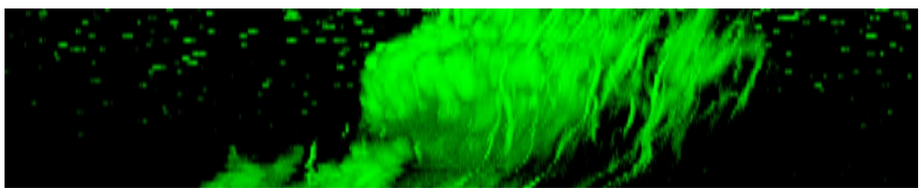

Fig. 4. The cross-section of the biofilm produced on the surface of the urological catheter after 48 hours of incubation, stained with SYTO-13 – *C. amycolatum* ATCC 700207 (above 50×).

Both the strains of *C. striatum* ATCC 6940 and *C. amycolatum* ATCC 700207 produced similar structures on the glass surface (Fig. 5). The biofilm on the glass surface formed small clusters and differed from the biofilm on the catheters. The positively charged surface of the glass did not contribute to the formation of the biofilm by *C. striatum* and *C. amycolatum* in contrast to the negatively charged surface of the catheters.

**The effects of ciprofloxacin on biofilm – the confocal microscope image analysis.** The effects of ciprofloxacin used in various concentrations on the biofilm produced by *C. striatum* ATCC 6940 were shown in the microscopic image (Fig. 6).

The area of dead cells (red) became progressively bigger along with the increasing doses of the antibiotic in comparison with the green-stained living cells of the biofilm. Yet, a visual inspection of the image under the microscope did not disclose the results that appeared in the drawings based on a statistical evaluation of the biofilm morphology measurements with the number of maxima. Figure 7 shows statistically significant differences of the area occupied by live bacteria for MIC6

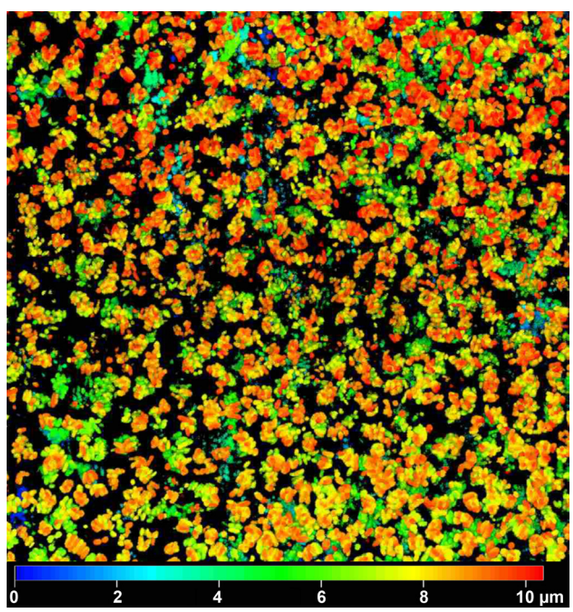


Fig. 5. The biofilm produced on the surface of the glass after 48 hours of incubation, stained with SYTO-13 with a marked height of the visible structures *C. striatum* ATCC 6940 (above 50×).

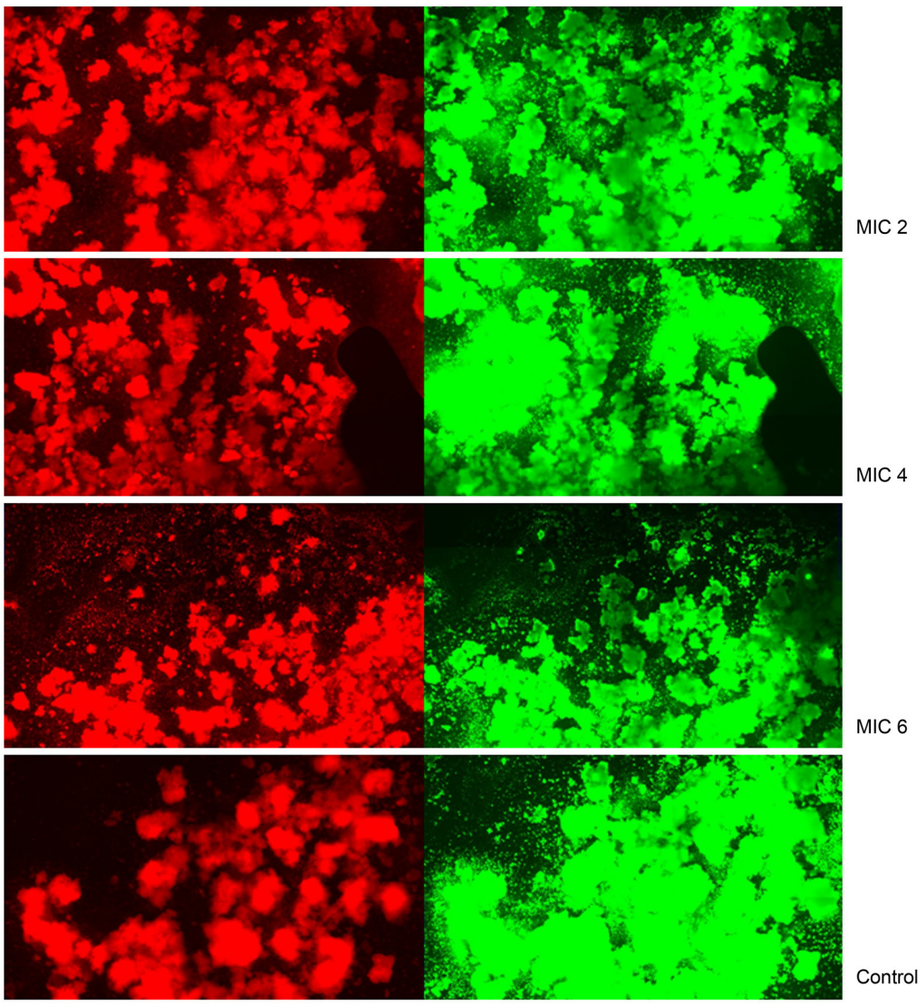


Fig. 6. Biofilms exposed to ciprofloxacin (MIC2 = 0,25 μg/l; MIC4 = 0,5 μg/l; MIC6 = 0,75 μg/l and control samples without antibiotic) – biofilm surface appearance: dead bacterial cells (red) and live bacterial cells (green) (above 200×).

against the control biofilm. In contrast, the percentage of biofilm surface containing live bacteria after treatment with ciprofloxacin MIC4 was higher than after treatment with the MIC2 concentration what was indicated by "flattenings" and the formation of a biofilm on a larger area compared to the control culture (Fig. 8 and Fig. 9). It can be assumed that a detachment of dead bacteria and the unveiling of deeper areas of biofilm live bacteria occurred as a result of the antibiotic activity, which was also confirmed by the values gained by the ratio of the area occupied by live bacteria to the area of dead bacteria after treatment with ciprofloxacin (Fig. 9). Paradoxically, the biofilm exposed to ciprofloxacin at a concentration equal to 4×MIC demonstrated the extension of the area in spite of the greater concentration of the antibiotic. It was also confirmed by the ratio of luminosity of living bacteria to dead bacteria (Fig. 10).

## Discussion

The ability of opportunistic *Corynebacterium* species to produce biofilm has already been demonstrated in numerous articles (Soriano et al 1993; Gomes et al. 2009; Frolova et al. 2014) on the other biofilm-producing microbes, such as: *Staphylococcus* spp., *Enterococcus* spp., *Streptococcus* spp., *Micrococcus* spp., which often accompanied infections associated with *Corynebacterium* (Cordero-Ampuero et al. 2007; Dowd et al. 2008; Kania et al. 2008; Wolcott et al. 2009). A special organs and tissues where the biofilm is developed and *Corynebacterium* spp. participates in mixed infections are surgical wounds (Kathju et al. 2009), sinuses in chronic sinusitis that may contain rare species, e.g. *Corynebacterium argentoratense* (Kania et al. 2008), and periprosthetic knee and joint infections (Cordero-Ampuero et al. 2007). Lipophilic bacteria on the skin,

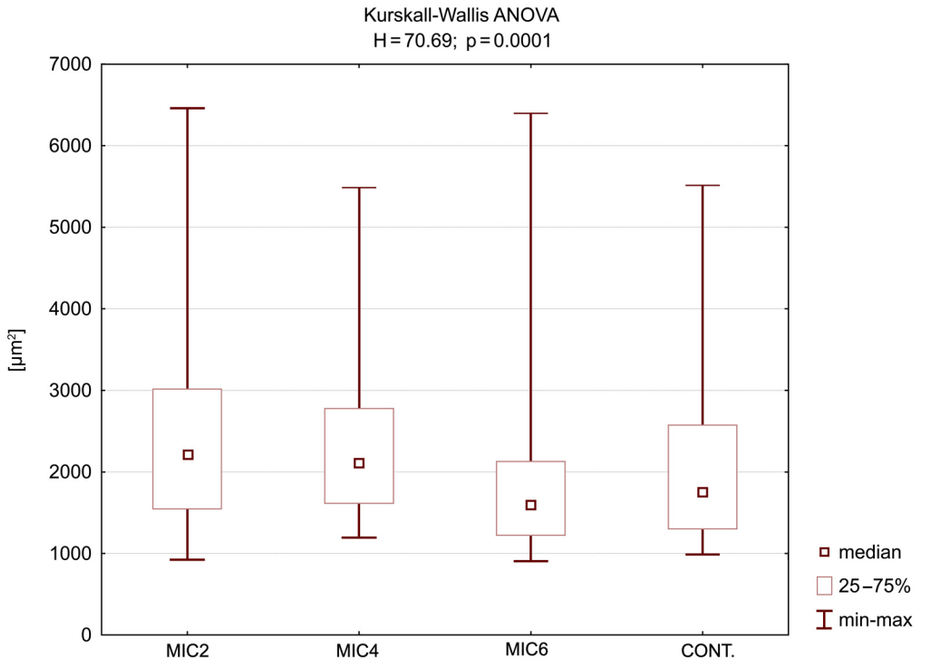


Fig. 7. The area occupied by the live bacteria forming the biofilm after treatment with ciprofloxacin at three concentrations (MIC2 = 0,25 µg/l; MIC4 = 0,5 µg/l; MIC6 = 0,75 µg/l) and control samples without the antibiotic.

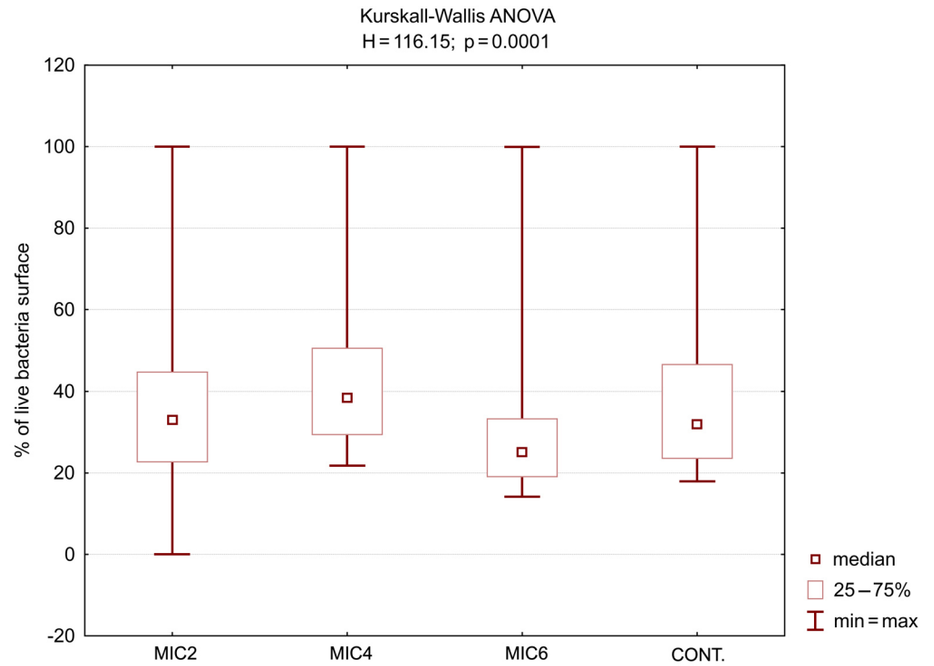


Fig. 8. The percentage of the biofilm surface with live bacteria after exposure to ciprofloxacin at three concentrations (MIC2 = 0,25 µg/l; MIC4 = 0,5 µg/l; MIC6 = 0,75 µg/l) and control samples without the antibiotic.

especially *Corynebacterium jeikeium* (Kwaszewska et al. 2006) are also involved in the development of multi-species biofilms.

The complex nature of biofilm formed by *Corynebacterium* spp. is confirmed by its detection on the surface of polyps in the nose along with other species found in the oropharynx (Zernotti et al. 2010).

Moreover, Gomes et al. (2009) reported the biofilm development by a nontoxic strain of *Corynebacterium diphtheriae* on the surface of polyurethane catheter

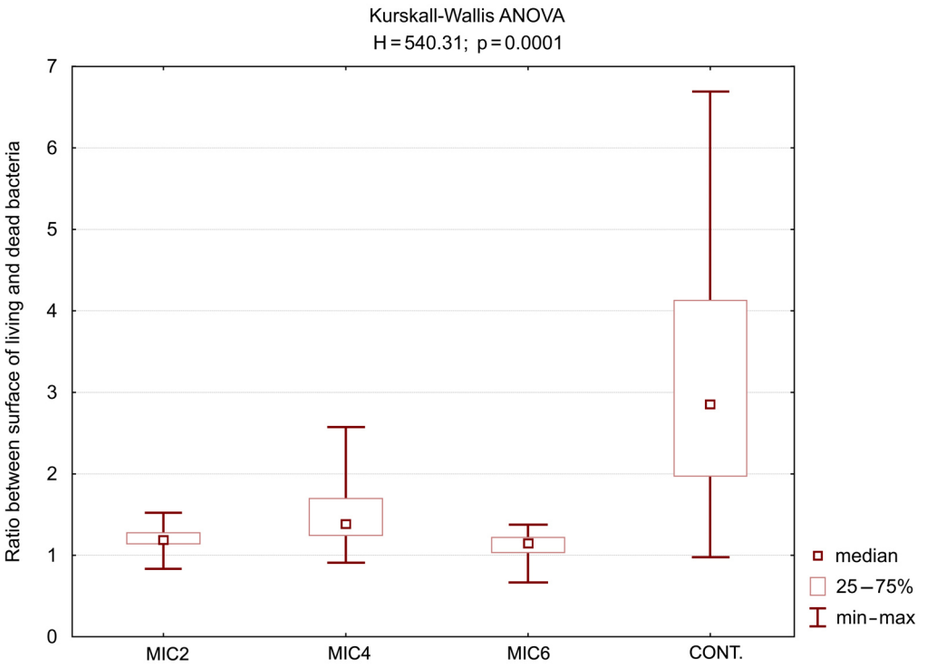


Fig. 9. The ratio of the area occupied by the live bacteria to the surface of dead bacteria within the biofilm after treatment with ciprofloxacin at three concentrations (MIC2 = 0,25 μg/l; MIC4 = 0,5 μg/l; MIC6 = 0,75 μg/l) and control samples without the antibiotic.

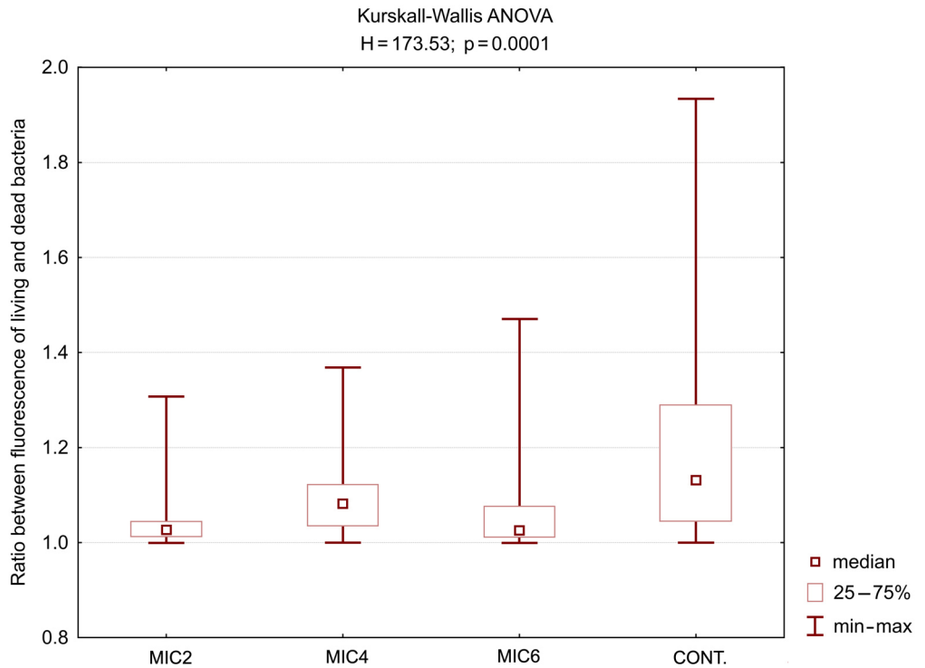


Fig. 10. The ratio of light intensity of live bacteria to dead bacteria after treatment with ciprofloxacin at three concentrations (MIC2 = 0,25 μg/l; MIC4 = 0,5 μg/l; MIC6 = 0,75 μg/l) and control samples without the antibiotic.

in a patient with cancer. The *in vitro* studies on the biofilm did not show high capacity of the *C. diphtheriae* strain to develope biofilms on polystyrene; they were formed more intensely on the glass surface. This strain also showed aggregation and adherence to the surface of HEp-2 cells.

One of the goals of this study was to develop an experimental model corresponding to *in vivo* conditions

during biofilms formation by *Corynebacterium* spp. on the catheter surface. Fragments of hospital vascular and urinary catheters were used in the research. The reference strains, representative of the species responsible for most infections caused by diphtheroids (*C. striatum* and *C. amycolatum*) were used in the experiments. These strains formed biofilms that differ in their structures and cross-sections, depending on the type of catheter. It results not only from the properties of the strains themselves, although this difference was also visible, but also from different chemical structure of the catheter surfaces (silicone, polyurethane). The image of the biofilm in the confocal microscope clearly draws attention to the compact nature of the bacterial mass in the cross-section of the vascular catheter, in comparison to clear fiber nature with visible water channels on the urinary catheter with its tendency to tear up.

An appropriate eradication method of the arising biofilm should be based on the knowledge of effects of various antimicrobial agents on its structure. Biofilms' important property is low susceptibility to antibiotics as well as to antiseptics and disinfectants, which makes the structure of the biofilm very difficult to destroy during the antibiotic therapy (König et al. 2002; Bridier et al. 2011; Williams et al. 2012).

*Corynebacterium urealyticum*, known for its potential to cause urinary tract infections, especially in catheterized and long-term hospitalized patients, has large capacity for adherence. Soriano et al. (2009) studied the biofilm produced by this species and investigated the effects of antimicrobial agents (ciprofloxacin, moxifloxacin, vancomycin and erythromycin) on biofilms produced in the "artificial urine" (formulation based on tryptic soy broth) by three strains of *C. urealyticum.* The MIC values of antibiotics recorded in this research were 2–8 times higher for the biofilm than for the planktonic forms. The study confirmed that only high concentrations of antibiotics could destroy the biofilm.

Different methods were used to determine the effects of antibiotics on biofilms in the *in vitro* assays. They were often associated with technical constraints related to the specific way in which biofilm were formed (Donlan 2002). The sensitivity to antibiotics was detected using the minimum inhibitory concentration by a method with serial dilutions technique of liquid growth medium (Frolova et al. 2014).

The investigation of antibiotic's effects on biofilms with the use of techniques that could damage their structure (e.g., sonication) may lead to misinterpretations, since it provokes bacteria to change the nature of their growth, shifting the cells (of the disintegrated biofilm) to the metabolism of vegetative forms (incubation in liquid media).

In this study, a method that did not damage biofilm was applied, and experiments were performed with the use of a confocal microscopy. This experimental model imitated the natural conditions. The statistical development of the data enabled a more accurate comparison of results.

The image acquired in confocal microscopy can also be very helpful in the direct diagnostics of the biofilm formed on the mucous membranes, e.g., in chronic sinusitis (Psaltis et al. 2007). To obtain detailed information on how the biofilm surface changes under the influence of ciprofloxacin, the evaluation of its effects was performed based on parameters of the biofilm such as: the size of the area occupied by live and dead bacteria and the intensity of luminosity of fluorescent dyes bound to bacteria. The later may be the indicator of altered cell surfaces after contact with the antimicrobial agent.

The statistical analysis of the results obtained here led to interesting conclusions. Namely, the number of live bacteria within the biofilm produced by *C. striatum* decreased, but paradoxically, its size and surface increased and the biofilms became flatter (compare Fig. 6 and Fig. 7).

The evaluation of the biofilm surface using the confocal microscopy was possible owing to the multiple scanning of successive areas of the surface examined. This approach to the analysis of the whole biofilm area allowed for the statistical analysis when the settings of the image were based on a constant value of the focus.

To the best of our knowledge, the method and microscope images analysis used in this study for investigation of the activity of antimicrobial agents on biofilms produced by opportunistic *C. striatum* ATCC 6490 has not been previously published. Moreover, this method could also be applied to investigate the biofilms of other bacterial species and their capacities to survive in the presence of antimicrobial agents.

## Conclusions

The *in vitro* evaluation of the ciprofloxacin effect on the biofilm produced by *C. striatum* ATCC 6490 revealed that its potential destruction would require an application of the antibacterial agent at a concentration higher than MIC6. Ciprofloxacin at a concentration equal to the value MIC4 caused a paradoxical increase of the surface of live bacteria within the biofilm. This suggests that the use of low doses of antibiotics may promote formation of a higher quantity of live bacteria in the biofilm. It could, in turn, promote detachment of planktonic bacteria from the biofilm structure and lead to their spread into the bloodstream or the neighboring tissues.

The applied method provides the original and novel approach to the investigation of the diphtheroid biofilms and their interaction with antimicrobial agents.

Moreover, the development of the method useful for comparison of the effects of changes in the biofilm structure under the influence of antimicrobial agents would also be helpful to study the properties of biofilms produced by other bacterial species and to investigate options of their eradication.

**Conflict of interest**

Authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

# Culturable Endophytes Diversity Isolated from *Paeonia ostii* and the Genetic Basis for Their Bioactivity

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Abstract

*Paeonia ostii* is known for its excellent medicinal values as Chinese traditional plant. To date, the diversity of culturable endophytes associated with *P. ostii* is in its initial phase of exploration. In this study, 56 endophytic bacteria and 51 endophytic fungi were isolated from *P. ostii* roots in China. Subsequent characterization of 56 bacterial strains by 16S rDNA gene sequence analysis revealed that nine families and 13 different genera were represented. All the fungal strains were classed into six families and 12 genera based on ITS gene sequence. The biosynthetic potential of all the endophytes was further investigated by the detection of putative polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) genes. The PCR screens were successful in targeting thirteen bacterial PKS, five bacterial NRPS, ten fungal PKS and nine fungal NRPS gene fragments. Bioinformatic analysis of these detected endophyte gene fragments facilitated inference of the potential bioactivity of endophyte bioactive products, suggesting that the isolated endophytes are capable of producing a plethora of secondary metabolites. These results suggest that endophytes isolated from *P. ostii* had abundant population diversity and biosynthetic potential, which further proved that endophytes are valuable reservoirs of novel bioactive compounds.

Key words: *Paeonia ostii*, endophytes, diversity, polyketide synthase, nonribosomal peptide synthetase

## Introduction

Endophytes are bacterial (including actinomycetes) or fungal microorganisms that spend part of or their entire life cycle inter- and/or intra-cellularly, colonizing healthy tissues of host plants without manifesting apparent symptoms of disease (Wani et al. 2015). These microorganisms produce numerous novel bioactive products, such as antibiotics, anticancer reagents, biological control agents, and other useful bioactive compounds for medical, agricultural, and industrial uses (Nisa et al. 2015; Venugopalan and Srivastava 2015). Thus, researchers have shown interest in bio-prospecting of endophytic microbial communities inhabiting plants from different ecosystems.

Medicinal plants are rich sources of precious bioactive compounds. Given their long-term association with each other, medicinal endophytes participate in metabolic pathways of medicinal plants, thereby producing analogous or novel bioactive compounds (Egamberdieva et al. 2017). Endophytic fungi of medicinal plants provide various bioactive secondary metabolites with unique structures; these metabolites include alkaloids, benzopyranones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, and xanthones (Aly et al. 2011; Deshmukh et al. 2015). Meanwhile, endophytic bacteria are presumed capable of producing a wide range of undescribed metabolites. Novel antibiotics produced by endophytic bacteria of medicinal plants include ecomycins, pseudomycins, munumbicins, and kakadumycins. Therefore, endophytes of medicinal plants are considered potential sources of novel bioactive compounds.

Recent studies have demonstrated that active compounds of endophytes can be categorized under two classes, namely, polyketides and nonribosomal peptides, which are biosynthesized by polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) systems (Amoutzias et al. 2016). A typical modular PKS is composed of acyltransferase (AT), ketosynthase

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(KS), and acyl carrier protein domains (Crawford et al. 2009). KS domain condenses an extender unit onto the growing polyketide chain during polyketide biosynthesis and is involved in production of structurally diverse metabolites. Encoded NRPSs are composed of multiple modules. Each module consists of an adenylation (A) domain, which facilitates selection and activation of amino acids; a peptidyl carrier protein domain; and a condensation (C) domain, which catalyzes peptide bond formation between two amino acids (Strieker et al. 2010). Each module spans 1000–1100 amino acids long; according to collinearity rule, the number and order of modules define the number and order of amino acids in NRPs, respectively (Nikolouli and Mossialos 2012).

Recent advances in development of bioinformatics tools have enabled application of functional gene-based molecular screening strategy for rational selection of endophytes; such biosynthetic screening strategy offers promising potential for targeted drug discovery. Genes for PKSs and NRPSs are among the most intensively encoded in endophytic microbial genomes. PKS and NRPS pathways are suitable targets for functional gene-based screening because of their roles in biosynthesis of many bioactive microbial metabolites (Evans et al. 2011). Using a PKS gene-based molecular screening strategy, researchers have discovered a new polyketide from endophytic fungi isolated from *Salicornia herbacea* containing PKS I genes (Wang et al. 2014). Therefore, encoding biosynthesis genes of PKSs and NRPSs in endophytes can be used as indicator in screening different endophytic strains to isolate novel polyketides and nonribosomal peptides.

Moutan cortex, which is the root bark of the medicinal tree peony *Paeonia ostii*, contains numerous pharmacological compounds with anti-microbial, anti-inflammatory, and anti-cancer activities (Lau et al. 2007). Various compounds that have been identified in the Moutan cortex include paeonoside, paeonolide, apiopaeonoside, paeoniflorin, oxypaeoniflorin, benzoyloxypaeoniflorin, benzoylpaeoniflorin, paeonol, and sugars (Chen et al. 2006a). Although considerable research effort has been devoted to biodiversity of endophytic microorganisms associated with medicinal plants, limited studies have explored the diversity and biosynthetic potential of endophytes in the medicinal tree peony. Thus, we performed 16S rRNA and ribosomal internal transcribed spacer (ITS) gene sequence analysis to investigate species diversity of culturable endophytic bacteria and fungi of *P. ostii* collected from Henan Province, China. We used degenerate primers of PKS and NRPS genes to screen the potential capacity of endophytes to synthesize secondary metabolites. The objective of this endeavor is to bio-prospect endophyte resources that can be potentially applied in pharmaceutical and agricultural fields.

## Experimental

### Materials and Methods

**Sample collection and endophyte isolation.** Samples for endophyte isolation were collected in April 2016 from a *P. ostii* plantation within and surrounding Luoyang City (34°43′ N, 112°24′ E). The area is characterized by a temperate monsoon climate with a mean annual temperature of 14.9°C and mean annual rainfall of ≈ 530 to 600 mm. The soils of this area are cinnamon soils derived from carbonatite. The fifty individual plants, and each of plants, representing about 10-year growth, were selected randomly. The plant root tissues were dug, transferred into sterile biosafety bags, and transported to the laboratory for further analysis. The samples were washed in running tap water to remove the clays on the surface of root tissue and checked for disease symptoms or superficial damage. Symptom-free root samples were then were separated into 10 pieces, and each of pieces, containing 5 g root tissues. Surface sterilization was performed on 2–3 cm tissue samples according to methods described by Guo et al. (2003). The samples were sterilized by washing with 70% ethanol for 1 min, followed by immersion in 3% sodium hypochlorite for 2 min and rinsing with 70% ethanol for 30 s. Disinfected samples were washed with sterilized water for five times and then drained (Potshangbam et al. 2017).

To isolate endophytic bacteria, surface-sterilized root segments were ground by pestle and mortar with 3 ml phosphate-buffered saline buffer (pH 7.0). Then, 100 µl aliquots of suspension were plated on tryptic soy agar (TSA) medium and Luria-Bertani (LB). Plates were incubated at 28°C for 3–5 days. Single colonies displaying different morphological characteristics were selected and stored in 15% glycerol at −80°C for further analyses. To isolate endophytic fungi, root tissues were cut into 0.5 cm pieces and placed on potato dextrose agar (PDA) medium. Five plant fragments were placed on each plate. In total, 280 root segments from 50 individual *P. ostii* plants were investigated. Plates were incubated at 25°C for 5–20 days in the dark and checked regularly. A mycelium from the colony margin was transferred to a new petri dish containing PDA medium to achieve single conidial selection. To determine successful sterilization, sterile distilled water used in the final rinse was plated on fresh TSA, LB and PDA plates, incubated at 28°C for 7 days, and then examined for any remaining epiphytic microorganism.

**Identification of endophytic bacteria and fungi.** The endophytic bacteria were identified according to the analysis of 16S rDNA. The endophytic fungi were identified according to morphology of the fungal culture, and the internal transcribed spacer sequences of nuclear ribosomal DNA (ITS1-5.8S-ITS2 rDNA

Table I
PCR primers for identification and screening the biosynthetic genes of endophytic isolates.

| Primer name | Primer Sequence (5'-3') | Target gene | Product size (bp) | References |
|---|---|---|---|---|
| 24F | AGAGTTTGATC(A/C)TGGCTCAG | 16S rDNA | 1400–1500 | Heuer et al. 1997 |
| 1492R | TACGG(C/T)TACCTTGTTACGACTT | | | |
| ITS1 | TCCGTAGGTGAACCTGCGG | ITS | 500 | White et al. 1990 |
| ITS4 | TCCTCCGCTTATTGATATGC | | | |
| KSαF | TSGCSTGCTTGGAYGCSATC | Bacterial PKS | 600–700 | Metsä-Ketalä et al. 1999 |
| KSαR | TGGAANCCGCCGAABCCGCT | | | |
| A3F | GCSTACSYSATSTACACSTCSGG | Bacterial NRPS | 700 | Ayuso-Sacido and Genilloud 2005 |
| A7R | SASGTCVCCSGTSGCG TAS | | | |
| KAF1 | GARKSICAYGGIACIGGIAC | Fungal PKS | 700–800 | Amnuaykanjanasin et al. 2005 |
| KAR1 | CCAYTGIGCICCRTGICCIGARAA | | | |
| AUG003 | CCGGCACCACCGGNAARCCHAA | Fungal NRPS | 600–700 | Slightom et al. 2009 |
| AUG007 | CCGGACCATGTCGCCNGTBYKRTA | | | |

sequence). Genomic DNA of endophytic isolates was extracted using Genomic DNA Kits (TaKaRa, China) in accordance with manufacturer's protocol. Bacterial and fungi isolates were identified by partial sequencing of 16S rDNA and ITS, respectively. Table I provides 16S rDNA and ITS gene primers used in this study. The 50 µl polymerase chain reaction (PCR) reaction mixture contained 100 ng of DNA extract, 1 × Taq reaction buffer, 20 pmol of each primer, 200 µM of each deoxynucleotide triphosphates, and 1.5 U of Taq DNA polymerase (Promega, USA). After initial denaturation at 94°C for 5 min, thermal cycling proceeded as follows: denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and elongation at 72°C for 1 min. After 35 cycles, the final extension step proceeded at 72°C for 10 min. PCR products were purified using TIANquick Midi Purification Kits (Tiangen, China) and sequenced at Sangon Biotech Co. Ltd (Shanghai, China). All obtained sequences were compared with those in the GenBank database by using the BLASTN search program. Similar sequences were further aligned by CLUSTALX (version 1.81). A phylogenetic tree was constructed based on evolutionary distance data by using MEGA software (version 5.0) (Tamura et al. 2011). Phylogenetic analysis was conducted by neighbor-joining method. Bootstrap analysis was performed with 1000 replications to determine the support for each clade.

**Detection and analysis of biosynthetic genes PKS and NRPS.** Four sets of degenerate primers targeting genes encoding PKS and NRPS were used to screen biosynthetic potential of bacterial and fungal isolates. Utilized primers are listed in Table I. PCR reaction mixture was the same as that described in the previous section. Genes were amplified using the following protocol: a denaturation step at 94°C for 5 min; 35 amplification cycles at 94°C for 1 min; 57°C (for A3F-A7R), 58°C (for KSαF-KSαR), or 60°C (for KAF1-KAR1 and AUG003-AUG007) for 1 min; 72°C for 2 min; and a final extension at 72°C for 5 min. Degenerate PCR products containing multiple sequences were cloned using pGEM®-T Easy cloning kits for sequencing (Promega, USA) and transformed into *Escherichia coli* DH5α. A clone library was established in accordance with manufacturer's instructions. Transformants were screened under standard PCR conditions and sequenced at Sangon Biotech Co. Ltd (Shanghai, China). To perform PKS and NRPS gene fragment analysis, an ExPASy translation tool was employed to identify amino acid sequences from DNA sequences (Gasteiger 2003). Amino acid substrates, which were recognized by the A domain binding pockets, were predicted using the NRPSpredictor analysis tool available at NRPSpredictor2 (http://nrps.informatik.uni-tuebingen.de/Controller?cmd=SubmitJob) (Rottig et al. 2011). All the obtained amino acid sequences were compared with those in the GenBank database by using the BLASTP search program. Similar sequences were further aligned by CLUSTALX, and a phylogenetic tree was constructed based on evolutionary distance data by using MEGA software (version 5.0).

## Results

**Isolation of endophytic bacteria and fungi from *P. ostii* root tissues.** Healthy *P. ostii* root tissues were used for endophyte isolation. No colonies emerged after the final rinsing in sterilization, suggesting that surface sterilization was effective, and that subsequent isolates were endophytes. Colony morphology of endophytic isolates on TSA, LB and PDA media indicated that 56 bacteria and 51 fungi isolates were obtained, implying that a highly diverse range of bacterial and fungi endophytes was derived from root tissues of *P. ostii*.

Table II
The distribution of endophytic bacteria and fungi within *Paeonia ostii*.

| Genera | No. of isolates | Relative abundance (%) |
|---|---|---|
| **Endophytic bacteria** | | |
| *Streptomyces* | Md1-1, Md1-2, Md1-3, Md1-18 | 7.1 |
| *Promicromonosporaceae* | Md1-20 | 1.8 |
| *Microbacterium* | Md1-5, Md1-29 | 3.6 |
| *Citricoccus* | Md1-48 | 1.8 |
| *Bacillus* | Md1-4, Md1-6, Md1-8, Md1-9, Md1-10, Md1-11, Md1-12, Md1-13, Md1-15, Md1-16, Md1-17, Md1-19, Md1-21, Md1-22, Md1-24, Md1-25, Md1-26, Md1-31, Md1-33, Md1-35, Md1-36, Md1-39, Md1-37, Md1-41, Md1-42, Md1-43, Md1-44, Md1-45, Md1-46, Md1-50, Md1-51 | 55.4 |
| *Psychrobacillus* | Md1-27 | 1.8 |
| *Lysinibacillus* | Md1-14, Md1-30 | 3.6 |
| *Planococcus* | Md1-49 | 1.8 |
| *Xanthomonas* | Md1-23, Md1-28 | 3.6 |
| *Pseudomonas* | Md1-34 | 1.8 |
| *Serratia* | Md1-32, Md1-47 | 3.6 |
| *Enterobacter* | Md1-38, Md1-40, Md1-52, Md1-53, Md1-56 | 8.9 |
| *Lelliottia* | Md1-7, Md1-54, Md1-55 | 5.4 |
| **Endophytic fungi** | | |
| *Cylindrocarpon* | Mdf-3, Mdf-10, Mdf-13, Mdf-15, Mdf-36, Mdf-38, Mdf-43, Mdf-47 | 15.7 |
| *Fusarium* | Mdf-5, Mdf-6, Mdf-7, Mdf-8, Mdf-11, Mdf-18, Mdf-22, Mdf-23, Mdf-25, Mdf-28 | 19.6 |
| unclassified *Nectriaceae* | Mdf-26 | 2.0 |
| *Thelonectria* | Mdf-1, Mdf-17 | 4.0 |
| *Cephalosporium* | Mdf-4, Mdf-48 | 4.0 |
| *Leptosphaeria* | Mdf-2, Mdf-9, Mdf-12, Mdf-16, Mdf-20, Mdf-29, Mdf-33, Mdf-34, Mdf-37, Mdf-40, Mdf-46 | 21.6 |
| *Alternaria* | Mdf-27, Mdf-30, Mdf-31, Mdf-32, Mdf-39, Mdf-41, Mdf-42, Mdf-44, Mdf-45, Mdf-49, Mdf-50, Mdf-51 | 23.5 |
| *Acrocalymma* | Mdf-35 | 2.0 |
| *Cladosporium* | Mdf-14 | 2.0 |
| *Macrophomina* | Mdf-19 | 2.0 |
| *Phomopsis* | Mdf-24 | 2.0 |
| *Mucor* | Mdf-21 | 2.0 |

**Identification and phylogenetic analysis of endophytic bacteria and fungi.** Isolates were classified by performing DNA sequencing of 16S rDNA genes of bacteria. PCR products of all 56 bacteria isolates were obtained from 16S rDNA, generating approximately 1400–1500 bp gene fragments. Similarly, PCR products of all 51 fungi isolates were acquired from ITS, generating approximately 400–500 bp gene fragments. PCR products were sequenced and then analyzed by BLASTN. All isolates exhibited 96–100% similarity to sequences in the National Center for Biotechnology Information (NCBI) database. Sequences with the highest similarities were further aligned by CLUSTALX, and phylogenetic trees were constructed. The 16s rDNA and ITS gene sequences were deposited in the GenBank with accession numbers MF581407 – MF581462 and MF574221 – MF574271, respectively.

The resulting phylogenetic tree of bacterial isolates (Fig. 1) revealed that isolates and reference sequences were clustered according to established taxonomic orders, with high bootstrap support. All bacterial isolates from *P. ostii* roots belonged to three phyla (i.e., Actinobacteria, Firmicutes, and Proteobacteria), clustered into groups corresponding to six taxonomic orders (i.e., Streptomycetales, Micrococcales, Bacillales, Xanthomonadales, Pseudomonadales, and Enterobacterales), and further classified into nine families and 13 genera. Majority of the isolates were categorized under *Bacillus* (55.36%), followed by *Enterobacter* (8.93%) and *Streptomyces* (7.14%) (Table II). As the

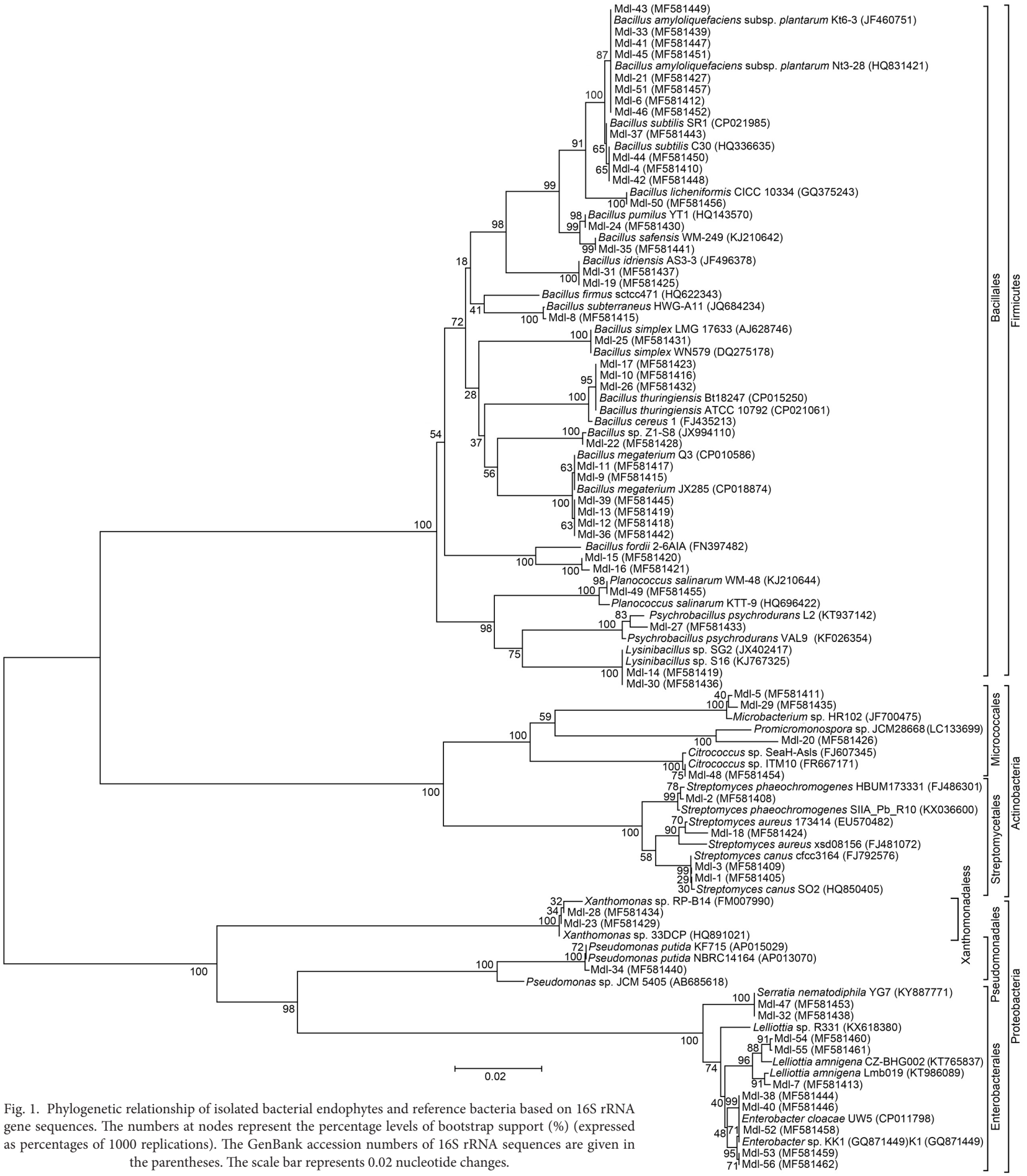


Fig. 1. Phylogenetic relationship of isolated bacterial endophytes and reference bacteria based on 16S rRNA gene sequences. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications). The GenBank accession numbers of 16S rRNA sequences are given in the parentheses. The scale bar represents 0.02 nucleotide changes.

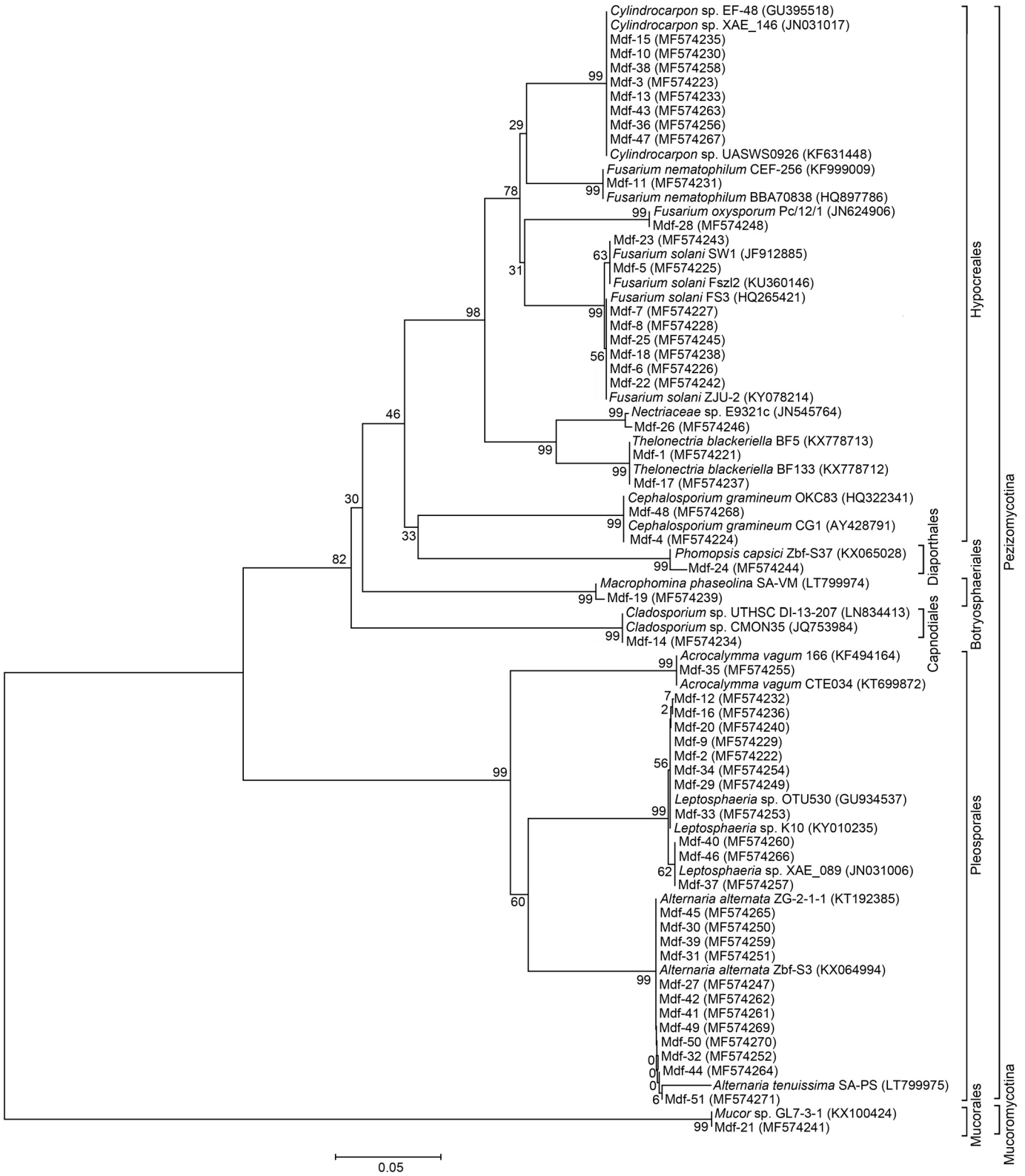


Fig. 2. Phylogenetic relationship of isolated fungal endophytes and reference fungal based on ITS gene sequences.
The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications).
The GenBank accession numbers of ITS sequences are given in the parentheses. The scale bar represents 0.05 nucleotide changes.

most abundant genus of isolates, *Bacillus* displayed high diversity of phylogenetically related species. Specifically for *Bacillus*, eight isolates showed an identical sequence to that of *B. amyloliquefaciens*, six isolates presented an identical sequence to that of *B. megaterium*, four isolates displayed an identical sequence to that of *B. subtilis*, and the other isolates exhibited identical sequences to those of *B. cereus*, *B. fordii*, *B. idriensis*, *B. subterraneus*, *B. licheniformis*, *B. pumilus*, *B. safensis*, *B. simplex*, and *Bacillus* sp. (Fig. 1).

The resulting phylogenetic tree of fungal isolates (Fig. 2) revealed that isolates and reference sequences were clustered according to established taxonomic orders, with high bootstrap support. According to phylogenetic tree, endophytic fungi obtained from *P. ostii* roots can be assigned to two different phyla (i.e., Pezizomycotina and Mucoromycotina). Pezizomycotina accounted for 98% of collected isolates, including Pleosporales (47.0% of total isolates), Hypocreales (45.0%), Capnodiales (2.0%), Botryosphaeriales (2.0%), and Diaporthales (2.0%). Majority of the Pleosporales were represented by genera of *Leptosphaeria* (21.6%) and *Alternaria* (23.5%). The second most abundant order was Hypocreales, which was dominated by *Fusarium* (19.6%) and *Cylindrocarpon* (15.7%) species. Mucoromycotina (*Mucor*) accounted for only 2.0% of total isolates (Table II).

**Screening of endophytic bacterial and fungal PKS and NRPS gene fragments.** Bacterial and fungal endophytes were surveyed for the presence of PKS and NRPS genes. Partial sequences of these complex biosynthesis genes were obtained from 15 out of 56 (27%) bacterial isolates, and positive results were achieved in 13 out of 56 (23%) and 5 out of 56 (9%) of the genetic screens for KS (PKS) and A (NRPS) domains, respectively. Three bacterial isolates, namely, Md1-2, Md1-24, and Md1-50, contained both putative PKS and NRPS genes (Table III). PCR screens of fungal endophytes revealed that 18 out of 51 (35%) fungal isolates contained either PKS (10 out of 51, 20%) or NRPS (9 out of 51, 18%). Only the funal isolate Mdf-41 contained both PKS and NRPS genes (Table IV). Sequences of bacterial PKS and NRPS genes and fungal PKS and NRPS genes were deposited in the GenBank with accession numbers MF589505-MF589517, MF589518-MF589522, MF680559-MF68568, and MF680550-MF680558, respectively (Table IV).

**Phylogenetic analysis of bacterial PKS and NRPS.** Using BLASTP query, nucleotide sequences of KS and A domain gene fragments were translated and

Table III
PKS and NRPS genes in endophytic bacteria isolated from *Paeonia ostii*.

| Gene | No. of isolates | Amino acid residues | Accession number | Top BLASTP match (GenBank accession No.) | Identity (%) | Predicted binding pocket (amino acid substrate) |
|---|---|---|---|---|---|---|
| PKS | Md1-2 | 226 | MF589505 | polyketide synthase, *Nostoc* sp. (AGJ72843) | 144/226(64%) | Not done |
| PKS | Md1-4 | 227 | MF589506 | polyketide synthase, *Bacillus* sp. (ACG70843) | 226/227(99%) | Not done |
| PKS | Md1-6 | 227 | MF589507 | type I ketosynthase, *Bacillus* sp. (AIO09656) | 220/224(98%) | Not done |
| PKS | Md1-9 | 224 | MF589508 | type I ketosynthase, *Bacillus* sp. (AIO09656) | 220/222(99%) | Not done |
| PKS | Md1-21 | 223 | MF589509 | type I ketosynthase, *Bacillus* sp. (AIO09652) | 222/222(100%) | Not done |
| PKS | Md1-24 | 227 | MF589510 | polyketide synthase, *Bacillus* sp. (ACG70842) | 224/227(99%) | Not done |
| PKS | Md1-37 | 227 | MF589511 | polyketide synthase, *Bacillus* sp. (ACG70841) | 226/227(99%) | Not done |
| PKS | Md1-41 | 227 | MF589512 | polyketide synthase, *Bacillus* sp. (ACG70841) | 226/227(99%) | Not done |
| PKS | Md1-43 | 226 | MF589513 | type I ketosynthase, *Bacillus* sp. (AIO09656) | 219/224(98%)) | Not done |
| PKS | Md1-44 | 224 | MF589514 | type I ketosynthase, Bacillus sp. (AIO09656) | 218/222(98%) | Not done |
| PKS | Md1-45 | 229 | MF589515 | polyketide synthase, *Bacillus* sp. (ACG70842) | 226/229(99%)) | Not done |
| PKS | Md1-50 | 227 | MF589516 | polyketide synthase, *Bacillus* sp. (ACG70843) | 227/227(100%) | Not done |
| PKS | Md1-51 | 223 | MF589517 | type I ketosynthase, *Bacillus* sp. (AIO09656) | 221/222(99%) | Not done |
| NRPS | Md1-2 | 232 | MF589518 | non-ribosomal peptide synthetase, *Streptomyces hiroshimensis*(BAH68742) | 158/217(73%) | DFECLSVVT-(Val) |
| NRPS | Md1-18 | 232 | MF589519 | non-ribosomal peptide synthetase, *Streptomyces hiroshimensis* (BAH68742) | 158/217(73%) | DFECLSVVT-(Val) |
| NRPS | Md1-24 | 252 | MF589520 | nonribosomal peptide synthase, *Bacillus* sp. (KIA75709) | 249/252(99%) | DAKDLGVVD-(Glu) |
| NRPS | Md1-47 | 233 | MF589521 | non-ribosomal peptide synthetase, *Pseudomonas* sp. (WP_085703687) | 225/233(97%) | DAWVFGVVI-(Glu) |
| NRPS | Md1-50 | 245 | MF589522 | non-ribosomal peptide synthetase, *Bacillus velezensis* (WP_069007535) | 243/245(99%) | DFWNIGMVH-(Thr) |

Table IV
PKS and NRPS genes in endophytic fungi isolated from *Paeonia ostii*.

| Gene | No. of isolates | Amino acid residues | Accession number | Top BLASTP match (GenBank accession No.) | Identity (%) | Predicted binding pocket (amino acid substrate) |
|---|---|---|---|---|---|---|
| PKS | Mdf-4 | 250 | MF680559 | related to fusarin C cluster-polyketide synthase/NRPS, *Rhynchosporium agropyri* (CZS94917) | 222/250(89%) | Not done |
| PKS | Mdf-15 | 238 | MF680560 | ketoacyl-synt-domain-containing protein, *Coniochaeta ligniaria* (OIW26903) | 201/240(84%) | Not done |
| PKS | Mdf-17 | 211 | MF680561 | beta-ketoacyl synthase domain-containing protein, *Metarhizium album* (KHO00577) | 201/240(84%) | Not done |
| PKS | Mdf-26 | 231 | MF680562 | PKS protein, *Trichoderma parareesei* (OTA00034) | 175/231(76%) | Not done |
| PKS | Mdf-41 | 234 | MF680563 | polyketide synthase PksF, *Alternaria alternata* (XP_018382155) | 233/234(90%) | Not done |
| PKS | Mdf-43 | 238 | MF680564 | ketoacyl-synt-domain-containing protein, *Coniochaeta ligniaria* (OIW26903) | 227/234(97%) | Not done |
| PKS | Mdf-44 | 234 | MF680565 | polyketide synthase PksF, *Alternaria alternata* (XP_018382155) | 131/217(60%) | Not done |
| PKS | Mdf-47 | 250 | MF680566 | related to fusarin C cluster-polyketide synthase/NRPS, *Rhynchosporium agropyri* (CZS94917) | 233/234(99%) | Not done |
| PKS | Mdf-49 | 234 | MF680567 | polyketide synthase PksF, *Alternaria alternata* (XP_018382155) | 233/234(99%) | Not done |
| PKS | Mdf-51 | 234 | MF680568 | polyketide synthase PksF, *Alternaria alternata* (AFN68297) | 233/234(99%) | Not done |
| NRPS | Mdf-2 | 244 | MF680550 | acetyl-CoA synthetase-like protein, *Stagonospora* sp. (OAK98265) | 218/244(89%) | No prediction |
| NRPS | Mdf-6 | 234 | MF680551 | nonribosomal peptide synthetase 1, *Neonectria ditissima* (KPM37793) | 195/235(83%) | DIGFVGGIF-(Ile) |
| NRPS | Mdf-8 | 231 | MF680552 | nonribosomal peptide synthetase 1, *Neonectria ditissima* (KPM37793) | 208/231(90%) | DVTLVGCVV-(Cys) |
| NRPS | Mdf-9 | 222 | MF680553 | nonribosomal peptide synthetase, *Cenococcum geophilum* (OCK98900) | 195/222(88%) | DVAFIGSIH-(Phe) |
| NRPS | Mdf-18 | 228 | MF680554 | nonribosomal peptide synthetase, *Cenococcum geophilum* (OCK98900) | 198/228(87%) | DVAFIGSIH-(Phe) |
| NRPS | Mdf-20 | 246 | MF680555 | acetyl-CoA synthetase-like protein, *Stagonospora* sp. (OAK98265) | 223/246(91%) | No prediction |
| NRPS | Mdf-22 | 238 | MF680556 | nonribosomal peptide synthetase 1, *Neonectria ditissima* (KPM37793) | 220/237(93%) | DAMLVGAVI-(Gln) |
| NRPS | Mdf-41 | 240 | MF680557 | nonribosomal peptide synthase, *Alternaria alternata* (XP_018382376) | 201/240(84%) | DAILVGAVV-(Gln) |
| NRPS | Mdf-50 | 238 | MF680558 | nonribosomal peptide synthetase 1, *Neonectria ditissima* (KPM37793) | 217/237(92%) | DAMLVGAVI-(Gln) |

compared with protein sequences in the NCBI database. As shown in Table III, bacterial KS domain fragments exhibited 64–100% similarity to database sequences. Phylogenetic analysis of putative PKS fragments validated BLASTP results and indicated that the majority of KS domain fragments are evolutionarily related to previously identified sequences. As illustrated in Fig. 3, the 12 bacterial PKS fragments amplified from *Bacillus* isolates were detected on a single clade. KS domain sequence of four isolates, namely, Md1-4 (*B. subtilis*), Md1-50 (*B. licheniformis*), Md1-24 (*B. pumilus*), and Md1-45 (*B. amyloliquefaciens*) was grouped with *B. subtilis* PksN and *B. velezensis* PksJ, which are involved in biosynthesis pathway of antibiotic bacillaene. KS domain fragments of five isolates (i.e., Md1-6, Md1-9, Md1-43, Md1-44, and Md1-51) were closely related and were clustered with the BaeN fragments involved in biosynthesis of bacillaene. PKS fragments Md1-37 and Md1-41 clustered within a clade containing *Bacillus* KS domains, which included a still uncharacterized KS domain from *Bacillus* sp. and *B. amyloliquefaciens*. KS domain sequence of Md1-2 was related to *Bacillus* sp. type I KS fragments. The remaining fragment, Md1-2, was amplified from a strain putatively identified as

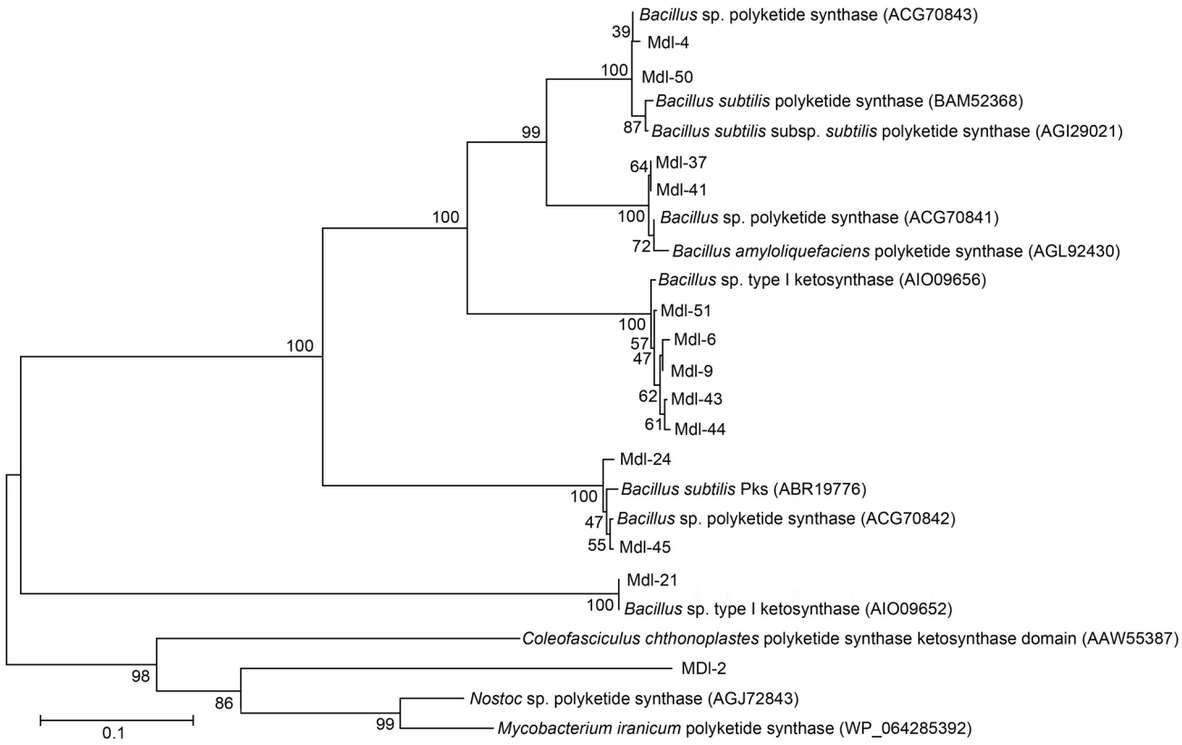


Fig. 3. The phylogenetic relationship of endophytic bacteria based on PKSs amino acid sequences homology. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications). The scale bar represents 0.1 amino acid changes.

*Streptomyces phaeochromogenes*. KS domain sequence of this strain was grouped with putative PKSs from *Nostoc* sp., which are cyanobacterial strains isolated from the Portuguese coast, and a PKS sequence amplified from a symbiont of nontuberculous mycobacterium species.

A domain fragments of bacterial endophytes displayed 73–99% similarity to A domain sequences in the database (Table III). The phylogenetic tree indicated that endophyte-derived fragments were similar to NRPS sequences from *Bacillus*, *Streptomyces*, and *Pseudomonas* in the database (Fig. 4). Sequences amplified from putative *Bacillus* isolates were phylogenetically related to NRPSs of *Bacillus* sp. (Md1-24 and Md1-50); these findings were consistent with those of BLASTP analysis. A domain sequence of Md1-24 was involved in biosynthesis of surfactin, which is a bacterial cyclic lipopeptide. The isolate Md1-50 was identified as *B. licheniformis* and clustered with the NRPS involved in producing siderophore bacillibactin. The A domain sequences of two isolates, namely, Md1-2 (*S. phaeochromogenes*) and Md1-18 (*Streptomyces aureus*), were grouped with NRPS fragments of *Streptomyces hiroshimensis*. *Streptomyces*-related NRPSs were involved in synthesis of numerous bioactive compounds. Another fragment of interest, Md1-47, was related to NRPS sequences from *Pseudomonas*.

Translated A domain sequences were further analyzed to determine whether they contained an eight-residue binding pocket and identify the amino acid that possibly binds to the pocket (Table III). NRPS sequences were successfully predicted for bacterial isolates, including Md1-2 and Md1-18, which possessed the same amino acid-binding pocket and can bind the Val residue. *Bacillus* isolates Md1-24 and Md1-50 possessed different binding pockets and were thus responsible for adding different amino acids to the growing peptide chain. Analysis of fragments detected from endophytes of *Pseudomonas* Md1-47 suggested that the binding pocket may integrate a Glu residue into a nonribosomal peptide.

**Phylogenetic analysis of fungal PKS and NRPS.** Fungal KS domain fragments exhibited 76–99% similarity to the database sequences (Table IV). Translated PKS fragments were aligned with the reference PKS sequences, and a phylogeny tree was constructed. The resulting phylogram (Fig. 5) revealed that all amplified fungal KS domain sequences belonged to the clade of type I PKSs, which synthesize reduced PKs. Four fungal PKS fragments amplified from *Alternaria* isolates (Mdf-41, Mdf-44, Mdf-49, and Mdf-51) were clustered within a single clade and highly homologous with *Alternaria* PksF, which is involved in biosynthesis pathways

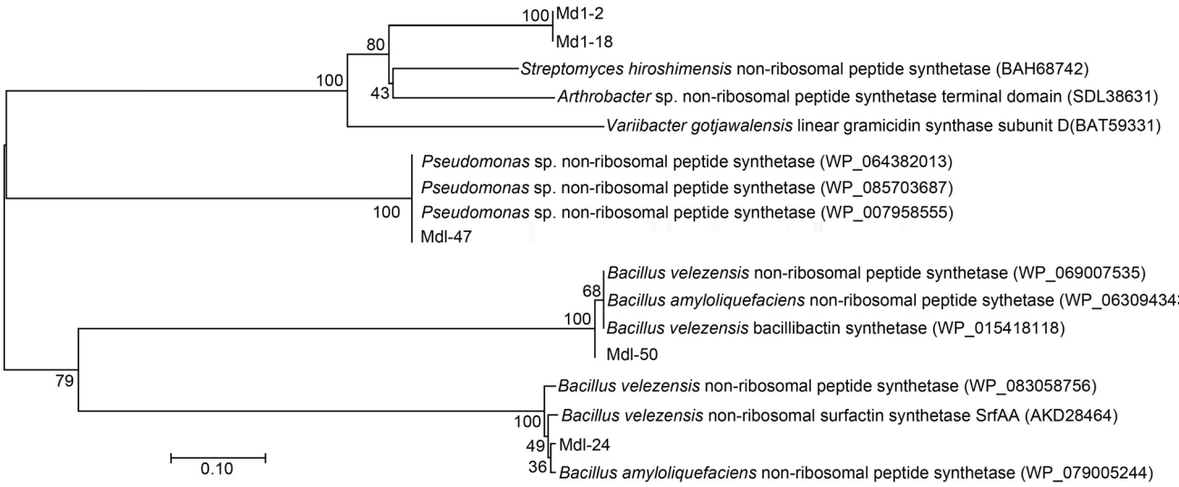


Fig. 4. The phylogenetic relationship of endophytic bacteria based on NRPSs amino acid sequences homology. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications).
The scale bar represents 0.10 amino acid changes.

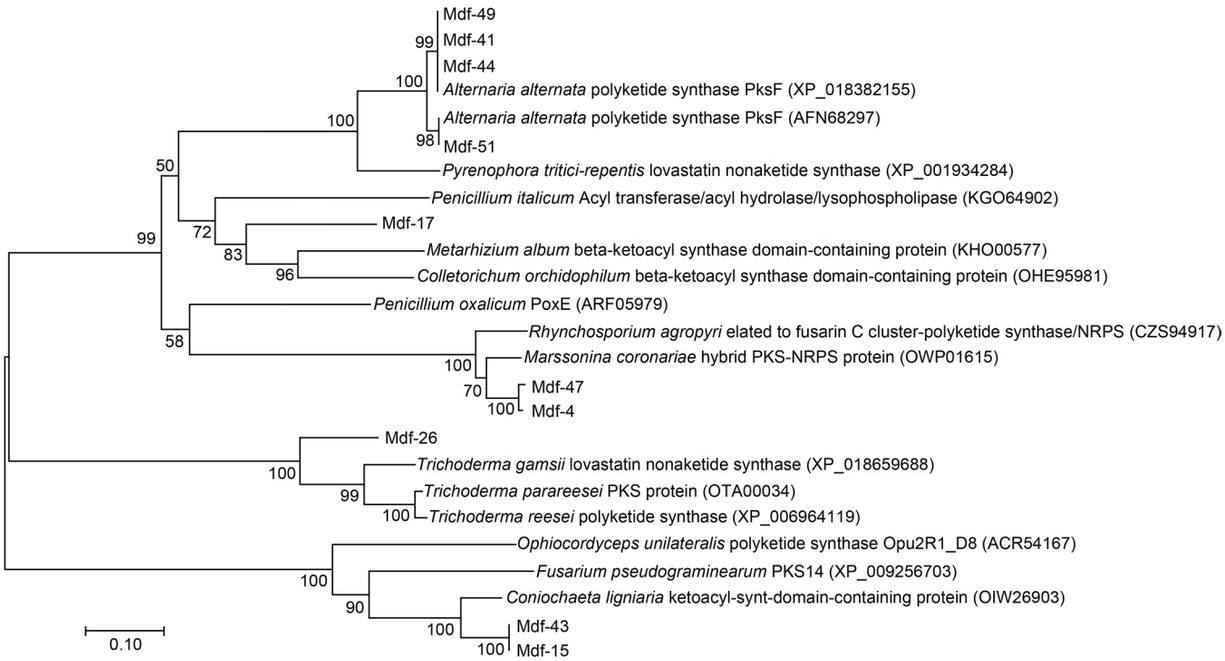


Fig. 5. The phylogenetic relationship of endophytic fungi based on type I PKSs amino acid sequences homology. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications).
The scale bar represents 0.10 amino acid changes.

of yellow pigment and two new polyene compounds. Isolates Mdf-4 and Mdf-47, which were closely related to each other, were clustered with hybrid PKS-NRPS fragments and showed slight similarity to PoxE fragments of *Penicillium oxalicum*. PKS fragments from endophytes of *Nectriaceae*, Mdf-26, were grouped with the lovastatin nonaketide synthase of *Trichoderma gamsii*. However, three PKS fragments (i.e., Mdf-15, Mdf-17, and Mdf-43) displayed slight similarity to fungal KS domain proteins, which were uninvolved in synthesis of active substances and therefore perform unknown functions.

Fungal endophyte A domain fragments showed 83–93% similarity to A domain sequences in the database (Table IV). The phylogenetic tree indicated similarity endophyte-derived fragments with NRPSs involved in synthesis of many bioactive compounds

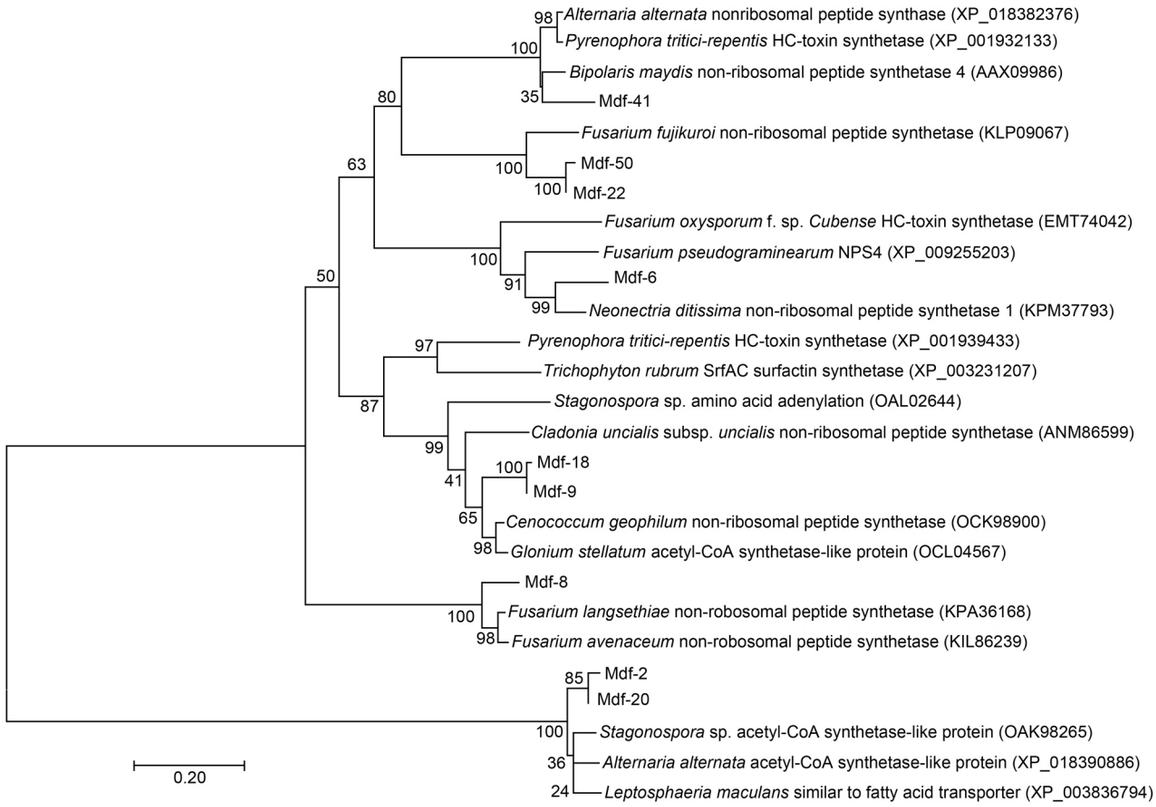


Fig. 6. The phylogenetic relationship of endophytic fungi based on NRPSs amino acid sequences homology. The numbers at nodes represent the percentage levels of bootstrap support (%) (expressed as percentages of 1000 replications). The scale bar represents 0.20 amino acid changes.

(Fig. 6). The A domain fragments of isolates Mdf-6, Mdf-22, Mdf-41, and Mdf-50 were clustered with a clade and were involved in biosynthesis pathways of fungal HC-toxin, NPS4, and NPS1. Isolates Mdf-9 and Mdf-18 were closely related to each other and showed slight similarity to the still uncharacterized NRPS fragments of *Cenococcum geophilum*. Gene products of Mdf-9 displayed similarity to putative NRPS fragments of *Fusarium*. The A domain fragments of Mdf-2 and Mdf-20 were clustered with a single clade and were highly homologous with the acetyl-CoA synthetase-like protein, which is involved in fungal biosynthetic and catabolic processes in *Stagonospora*.

The nine translated endophytic fungal NRPS fragments were analyzed to predict active residues of the A domain binding pocket. Seven amino acid binding pockets of fungal NRPSs were predicted successfully (Table IV). However, no NRPS amino acid binding pocket was detected in the other two strains (Mdf-2 and Mdf-20). Mdf-22, Mdf-41, and Mdf-50 showed the same amino acid binding pocket and bound to the Gln residue. Mdf-9 and Mdf-18 presented the same amino acid binding pocket and bound to the Phe residue. The remaining two isolates, Mdf-6 and Mdf-8, featured different amino acid binding pockets, and they integrated Ile and Cys residues into nonribosomal peptides, respectively.

## Discussion

Endophytes participate in long-term symbiotic relationships with their host plants, and many of them produce bioactive substances as consequence of these relationships. Structures of active compounds produced by endophytes are considered superior to those produced by their host plants. As such, endophytes, particularly those from medicinal plants, have become important sources of novel and biologically active secondary metabolites (Wang and Dai 2011). To aid this purpose, a more diverse and comprehensive collection of endophytes must be realized. Screening and isolation of promising strains of endophytes must be conducted to produce novel bioactive compounds for pharmaceutical and agricultural applications.

Tree peony (*P. ostii*) is a Chinese traditional plant considered valuable for its ornamental and medicinal benefits. However, limited information is available

regarding its endophytic community. This study investigated preliminary endophytes diversity from *P. ostii* and their potential ability to synthesize some secondary metabolites. Results showed abundance of a diverse range of endophytes in root tissues *P. ostii*. Endophytes comprised 56 bacterial endophytes classified under 13 different genera and 51 fungal endophytes categorized under 12 fungal genera. A large proportion of the endophytic bacteria and fungi possessed PKS and NRPS genes, suggesting that these endophytes are potential sources of bioactive substances.

Recent studies have explored endophyte diversity in medicinal plants, such as *Aloe vera* (Akinsanya et al. 2015), *Ferula songorica* (Liu et al. 2016), the medicinal cactus *Opuntia humifusa* (Silva-Hughes et al. 2015), and *Rhodiola rosea* (Cui et al. 2015). The most commonly found endophytes belong to the genera *Streptomyces* sp., *Bacillus* sp., *Pseudomonas* sp., *Enterobacter* sp., *Alternaria* sp., *Leptosphaerulina* sp., *Fusarium* sp., *Colletotrichum* sp., *Phomopsis* sp., *Phyllosticta* sp., and *Cladosporium* sp. (Nair and Padmavathy 2014).

Among the 56 endophytic bacteria obtained from *P. ostii*, *Bacillus* was the most dominant genus (55.4% of all isolates); this finding is consistent with previous reports on different hosts (El-Deeb et al. 2013). In addition to *Bacillus* sp., other genera, such as *Streptomyces* from *Schima wallichi* (Passari et al. 2016*)* and *Leifsonia* from ginseng roots, have also been reported in medicinal plants (Qiu et al. 2007). Endophytic *Bacillus* spp. isolated from plant tissues are highly abundant and considered microbial factories for numerous biologically active molecules that potentially inhibit phytopathogen growth (Ongena and Jacques 2008). Endophytic *Streptomyces* are considered potential sources of secondary metabolites and various bioactive products that exhibit antimicrobial, antioxidant, and plant-growth-promoting activities (Lam 2006; Nimnoi et al. 2010). These results indicated that tree peony acts as a reservoir of novel endophytic bacteria for isolation of biologically active compounds.

In this study, the dominant endophytic fungal genera in *P. ostii* belong to *Alternaria* (23.5%) and *Fusarium* (19.6%). *Alternaria* has been previously observed as the predominant fungal taxa in 29 traditional Chinese medicinal plants and a well-known medicinal plant in India (Huang et al. 2008; Gond et al. 2012). *Fusarium* was isolated as the dominant endophytic fungal species from five medicinal plant species in the Western Ghats of India (Raviraja 2005). Previous reports have also stated that *Alternaria* and *Fusarium* are common endophytes of tropical, subtropical, and temperate plants (Banerjee 2011; Gong et al. 2015). Colonization and propagation of these endophytes may offer significant benefits to their hosts by producing useful substances. Metabolites with antimicrobial activity have been found in endophytic fungi isolates from *Fusarium* and *Alternaria* (Brady and Clardy 2000; Raviraja et al. 2006).

Endophytes are presumed ubiquitous in the plant kingdom, and its population depends on host species and location. Previous studies have demonstrated that different host plants feature distinct endophyte community compositions, suggesting that endophytes display host preference (Cohen 2006). Studies have also presented significant differences in both the presence and absence and abundance of endophytes in other medicinal plants. For example, *Brevundimonas* and *Sphingomonas* are the dominant endophytic genera in *Ferula songorica* (Liu et al. 2016), whereas *Aspergillus* is the dominant fungal member in *Aegle marmelos* (Gond et al. 2012). These endophytic genera were not detected in *P. ostii* roots. By contrast, *Enterobacter* (8.93%), *Leptosphaeria* (21.6%), and *Cylindrocarpon* (15.7%) existed in *P. ostii* at high frequencies. Our results further validated specificity and selectivity between endophytes and host plants. Nonetheless, given the inadequate number of our samples, further investigations are needed to prove specificity and selectivity phenomena. Endophyte community is also dynamic, and species composition is affected by various factors (e.g., seasonal changes and host age); however, culture-independent endophytes were not considered in this study.

PKS and NRPS genes serve as appropriate targets for detecting small molecule biosynthesis systems (Moffitt and Neilan 2001; Sauer et al. 2002). Previous PKS and NRPS screening studies have also utilized these biosynthetic pathways to isolate endophytes from other terrestrial and marine environments (Zhou et al. 2011). In the present study, numerous PKS and NRPS genes were detected in endophytes from *P. ostii*. However, type I PKS and NRPS primers were used in this study. These primers cannot amplify all PKS or NRPS genes of endophytes, suggesting that these primers underestimated the diversity of KS and NRPS genes of endophytes from *P. ostii*. Therefore, future works should design new primers that can detect atypical PKS or NRPS genes of endophytes.

Twelve gene sequences detected in bacterial KS domain screen were amplified from isolates, which were identified to belong to the *Bacillus* genus. *Bacillus* spp. are prominent members of endophyte populations (El-Deeb et al. 2013), and they are known for their production of antibiotics, including subtilin, macrolactins, bacillaene, and difficidin (Mongkolthanaruk 2012). These KS domain fragments were grouped with *Bacillus* PksJ, PksN, and BaeN (Fig. 3). Dihydrobacillaene and bacillaene biosynthetic mechanism in *B. subtilis* is split among five large megasynthases (PksJLMNR) and several accessory proteins (Butcher et al. 2007). BaeN, subsequently termed PksN, is a secondary hybrid NRPS-PKS protein involved in biosynthesis of bacil-

laene (Chen et al. 2006b). The PKS gene amplified from *Streptomyces* Md1-2 was similar (64%) to PKS genes of *Nostoc* sp.; these cyanobacteria are prolific sources of bioactive products with interesting biological activities and promising pharmaceutical applications (Burja et al. 2001). PKS of *Nostoc* was mainly involved in biosynthesis of nostophycin and microcystins, and the two cyclic heptapeptides are potent inhibitors of eukaryotic protein phosphatases 1 and 2A (Fewer et al. 2011). Results implied that these putative KS domain genes from tree peony endophytes take part in synthesis of bacillaene and other antibiotics.

NRPS genes of bacterial isolates Md1-24 and Md1-50 were phylogenetically grouped with biosynthesis pathways of diverse *Bacillus* NRPS. The A domain genes of Md1-24 and Md1-50 were grouped with NRPSs of *B. velezensis*, which participate in synthesis of surfactin and bacillibactin synthetase (Fig. 4). Lipopeptides from *Bacillus* are synthesized by NRPS. As lipopeptide-type biosurfactants, surfactins are vital for some bacterial bioformation and root colonization, and they exhibit a wide range of antimicrobial activities (Bais et al. 2004; Aleti et al. 2015). Bacillibactin is catecholate-type siderophore produced by *Bacillus*; it serves as an iron scavenger for overcoming iron limitation. In recent times, endophytic bacterial siderophores have been applied in different fields, such as promoting plant growth of species by enhancing Fe uptake of plants and controlling phytopathogens as potential biocontrol agent (Saha et al. 2016). Other bacterial NRPS genes (Md1-2, Md1-18, and Md1-47) were similar to the still uncharacterized NRPS genes of *Streptomyces* and *Pseudomonas* (Fig. 4). Both *Streptomyces* and *Pseudomonas* are prolific producers of bioactive compounds, including various antibiotics, antitumor, and plant growth hormones (Wu et al. 2011; Passari et al. 2016). Therefore, these NRPS genes may be capable of producing unique bioactive compounds.

The presence of PKS I genes in fungi can increase chances of finding structurally novel polyketides with biological activities. In this study, all 10 KS domain fragments were within clusters of PKSs that synthesize reduced polyketides. Four putative fungal KS domain fragments from *Alternaria* spp. were closely related to *Alternaria alternata* PksF gene involved in biosynthesis of yellow pigment and two new polyene compounds, namely, aslanipyrone and aslaniol (Kasahara et al. 2006). Fungal pigments displayed a wide range of bioactivities; for example, elsinchrome possesses phytotoxic activities, whereas napthopyrones exhibits antimicrobial activities (Liao and Chung 2008). Detection of pigment and new polyene compounds posits biosynthetic potential of endophytes from *P. ostii*. Numerous polyketide compounds, such as lovastatin and fusarin, were also observed to contain the PKS I gene (Wang et al. 2014). Lovastatin, which was originally isolated from *Aspergillus terreus*, is widely used as a statin group in cholesterol-lowering agents. Fusarin, which is a broad-spectrum plant toxin, contributes to severity of plant vascular wilt induced by *Fusarium oxysporum*, damping-off, and root rot diseases (Hajjaj et al. 2001; Brown et al. 2012). Therefore, the presence of PKS gene in Mdf-4, Mdf-47, and Mdf-26 isolates implies their genetic potential to produce lovastatin or and fusarin-like metabolites. Other KS domain fragments, such as Mdf-15, Md1f-17, and Mdf-43, were related to the fungal KC domain protein involved in AT biosynthesis in the metabolic process. These amplified KS domains may participate in biosynthetic pathways, but functions relating to polyketide synthesis require further investigation.

Phylogenetic analysis of putative fungal A domains revealed that observed sequences were clustered into two main groups (Fig. 6). One group included seven isolates, namely, Mdf-6, Md1f-8, Mdf-9, Md1f-18, Mdf-22, Mdf-41, and Mdf-50, all of which were similar to fungal HC-toxin, NPS4, and NPS1 genes. NRPS can synthesize nonribosomal peptides, such as mycotoxins and HC-toxin (Bushley and Turgeon 2010). The HC-toxin produced by fungal plant pathogens generally act as effectors that control pathogenicity or virulence in certain plant-pathogen interactions (Tsuge et al. 2013). However, their functions in plants endophytic fungal remain unclear. Thus, further studies are needed to elucidate the potential role of toxin compounds in endophytes-host interactions. Recent studies on NPS genes of *Fusarium graminearum* revealed that NPS1 and NPS4 are related to genes involved in biosynthesis of NPS hydroxamate siderophores (Tobiasen et al. 2006). Therefore, the NRPS gene in endophytic fungi was presumed capable of producing siderophore compounds. The other group included two strains, namely, Mdf-2 and Mdf-20, which are similar to the fungal acetyl-CoA synthetase-like protein, which is utilized for fatty acid biosynthetic processes (Starai and Escalante-Semerena 2004).

In summary, population diversity of endophytes residing in roots of medicinal plant *P. ostii* was studied preliminary. Genetic screening revealed the presence of endophyte-derived PKS and NRPS fragments with putative roles in biosynthesis of secondary metabolites that present a wide array of bioactivities. Findings of this screening encourage future investigations to focus on links between PKS/NRPS genes and bioactivity to clarify full biosynthetic pathways of endophytes. Further studies are also required to determine whether the PKS and NRPS genes identified in this study are functionally dedicated to specialized endophyte-based activities and elucidate chemical components of bioactive metabolites produced by endophytes containing PKS/NRPS genes.

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**Conflict of interest**

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## Literature

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ORIGINAL PAPER

# Comparison of the Photosensitivity of Biofilms of Different Genera of Cariogenic Bacteria in Tooth Slices

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Abstract

This study compared the outcome of photosensitization on the viability of four different cariogens in planktonic form as well as biofilms in human dentine. Photodynamic therapy was carried out with a gallium aluminium arsenide laser (670 nm wavelength) using Toluidine blue O (TBO) as the photosensitizer. Cariogenic bacteria (*Streptococcus mutans*, *Lactobacillus casei*, *Streptococcus salivarius* and *Actinomyces viscosus*) were exposed to TBO and then to the laser for 1 minute in planktonic suspension. Then, tooth slices previously incubated for 24 hours with broth cultures of broth culture of the four cariogenic organisms were exposed to antimicrobial photosensitization. The control samples consisted of planktonic and sessile cells that were exposed to TBO alone, laser alone and the bacterial cells that were not treated with TBO or laser. The results showed significant reductions in the viability of *S. mutans*, *L. casei* and *A. viscosus* in both planktonic form (to 13%, 30%, and 55%, respectively) and sessile form hosted in dentinal tubules (to 19%, 13% and 52%, respectively), relative to the controls. *S. salivarius* was the least affected in planktonic (94% viability) and sessile form (86% viability). In conclusion, sensitivity to photosensitization is species-dependent and sessile biofilm cells are affected to the same extent as their planktonic counterparts.

Key words: cariogenic bacteria, planktonic, sessile cells, tooth slice, photodynamic therapy

## Introduction

Despite major advances in dentistry, caries persists as a very common infectious disease of children and adults, and one of a major public health problem (Ten Cate 2013). Many bacteria are present in the mouth and assemble into a mass of accumulated bacteria on the tooth surface in the form of dental plaque, i.e., dental biofilm. Cariogenic bacteria become part of the dental biofilm during early childhood and in due course proliferate under a favorable milieu to cause disease (Smith 2002).

Although there is extensive ongoing research into development of a vaccine to prevent dental caries, there is currently no satisfactory vaccine available. In the search for an alternative approach to conventional methods of caries elimination, the antimicrobial photodynamic therapy (PDT) is becoming a popular possible choice. Different studies have reported that planktonic cells of cariogenic bacteria are sensitive to eradication by PDT (Burns et al. 1994; Williams et al. 2003; Paulino et al. 2005; Metcalf et al. 2006; Tonon et al. 2015). It remains true, however, that the causal agents of caries and periodontitis exist in biofilms on the surface of the teeth or within the tooth structure itself once the carious lesions have been initiated. Furthermore, bacteria in biofilms may be 1000 times more impervious to the action of antimicrobial agents and host defense systems compared to planktonic suspensions (Welin-Neilands and Svensater 2007; Jakubovics and Kolenbrander

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2010), and thus the effect of PDT on oral or cariogenic bacteria in biofilms has also been investigated (Zanin et al. 2006; Lee et al. 2012; Mang et al. 2012).

Appreciable destruction of *S. mutans* has been reported when the organisms were in a milieu similar to carious teeth encapsulated in collagen or inside carious bovine teeth (Williams et al. 2004; Giusti et al. 2008). Lethal photosensitization could also be achieved when *S. mutans* was enclosed in collagen and irradiated with light that first travelled through demineralized dentine (Burns et al. 1995). Studies on the effectiveness of PDT on cariogenic bacteria present as biofilms in root canals and human dentine, although limited, have also been reported. Indeed, considerable elimination of biofilms of *S. intermedius* in root canals as well as *Escherichia coli and Enterococcus faecalis* in the extensive layers of dentine has been reported (Seal et al. 2002; Schoop et al. 2004).

Despite the fact that *S. mutans* plays a principal role in the induction of caries, many other microorganisms present in dental biofilms have additionally been implicated in the evolution of the lesions (Tanzer et al. 2001). These include *S. salivarius*, *S. sanguis*, *Lactobacillus casei* (progression of lesions) and *A. viscosus* (root surface caries) (Edwardsson 1987; Tanzer et al. 2001). Since the susceptibility of biofilms of different bacterial species to PDT may vary, the objective of the present study was to investigate whether different cariogenic bacteria present as biofilms in tooth slices are susceptible to photosensitization and to compare this susceptibility to that of the same bacteria in planktonic suspension using Toluidine blue O (TBO) as the photosensitizing agent activated by light from a laser diode.

## Experimental

### Materials and Methods

**Light source and photosensitizer.** Light for photosensitization was generated with a laser diode (gallium aluminium arsenide laser) (GaAlAs) (Q-beam 2001-A, Quantum devices Inc., Barneveld, Wisconsin, USA) with a central wavelength of 670 nm, which covered the absorbance of the photosensitizer, with an output power of 65W and photon flux density of 2000 μmol of photons $m^{-2}s^{-1}$. The distance between the bacterial suspensions or tooth slice specimens and the center of the GaAlAs laser was 13.5 cm, and appropriate spot sizes (1.5 $cm^2$) were made with an objective lens to cover the sample.

Toluidine blue O (TBO) (Sigma Ltd., Poole, UK) was used as the photosensitizer at a concentration of 10 mg/ml in distilled water.

**Bacterial strains and culture.** The cariogens consisted of: *S. mutans* (NCTC 10449), *L. casei* (NCTC 6375), *A. viscosus* (ATCC 43146) and *S. salivarius* (NCTC 8606). The bacteria were routinely cultivated on blood agar (Fluka Biochemica, Buchs, Switzerland). They were also cultivated in tryptone soya broth (Oxoid Ltd, Basingstoke, UK) and incubated for 24 h aerobically at 37°C and then used for the experiments at a density of $1 \times 10^7$ colony forming units/ml.

**Photosensitization of planktonic cultures.** Equal volumes of cultures of each of the four bacteria (triplicate samples) in tryptone soya broth were mixed with TBO (50 μg/ml) and incubated in the dark for 5 minutes. Then, they were exposed to laser light for 1 min, after which serial dilutions were prepared to determine bacterial viability. Aliquots of 100 μl from each dilution were plated onto blood agar and incubated at 37°C under aerobic conditions for 48 h, and then the number of visible colonies was counted. Cultures that had not been exposed to photosensitizer or laser were used to determine the total number of bacteria present in the cultures at the beginning of the experiment. The experiments were repeated three times and the average of each experiment was taken and the percentage viability of each culture was calculated relative to controls (controls consisted of cultures of bacteria without being exposed to photosensitizer or laser).

The cultures of the cariogenic bacteria exposed to the laser alone for 1 min and the bacterial cultures exposed to TBO for 6 min served as additional controls in order to evaluate any light toxicity and any toxicity from the photosensitizer, respectively.

**Preparation of tooth slices.** The teeth used in this study were third molars (wisdom teeth) extracted from adult patients who were 20 to 35 years of age. The teeth were vital but indicated for extraction due to the fact that they were malpositioned. The extracted molars were collected and stored in 0.2% thymol (Sigma Ltd., Poole, UK). The teeth were washed and cleaned with a brush using soap and water, and the roots of the teeth were removed and the buccal and lingual surfaces were then flattened to reach the dentine using a diamond disc on a slow-speed hand piece. Following this, the teeth were sliced in the middle from the mesial to the distal surface. The slices were approximately equal in shape and size ($10 \times 6 \times 3$ mm). The tooth slices were brushed again, and exposed to 17% EDTA, followed by 5.25% NaOCl, to remove the smear layer and washed with distilled water and autoclaved for 20 minutes and stored under aseptic conditions for later use.

**Photosensitization of bacteria present as biofilms in tooth slices.** The different cariogenic bacteria were cultured in tryptone soya broth for 24 h at a temperature of 37°C. The tooth slices were immersed in one ml suspensions of each of the bacterial strains in sterile test tubes. The tooth slices were incubated with the bacteria for 24 h at 37°C to allow the formation of biofilms

in the dentine. Bacterial suspensions were then aspirated and the tooth slices were washed once with distilled water (1 ml). Following this, 1 ml of TBO (50 μg/ml) was applied to the tooth slices and left for 5 min in the dark. For each bacterial strain nine tooth slices were exposed TBO and laser. Furthermore, nine tooth slices were exposed to laser only, another nine were exposed to TBO only, and the controls consisted of nine tooth slices that were exposed neither to laser nor TBO. Exposure to laser was carried out in the dark. The light emitted from the diode laser was focused onto the tooth specimen, which was inside the tube so that the whole tooth surface was exposed to the light. The tooth slices were illuminated for 1 min on each side. Following this, aliquots of fresh tryptone soya broth were added to each tube and the tooth slices were agitated using a vortex mixer. Serial dilutions were then prepared and 100-μl aliquots inoculated onto blood agar. The number of colonies was then enumerated and the percentage viability of each culture was calculated relative to controls (controls consisted of tooth slices without exposure to laser or TBO).

**Data and Statistical Analysis.** The effect of laser alone, TBO alone, and laser and TBO on the viability of the bacteria was determined relative to the controls (100% viability). Two-way analysis of variance (ANOVA) was used to determine any significant effects of the treatment protocol or bacterial species on the percentage viability of the cariogenic bacteria. Follow up comparison between the groups were then carried out using Tukey multiple comparison test ($\alpha = 0.05$).

## Results

Figures 1–4 show the results of exposure of both planktonic and sessile biofilm cells of *S. mutans*, *L. casei*, *A. viscosus*, and *S. salivarius* to laser, TBO, and laser with TBO. The results show that there was little direct toxicity with TBO as a sensitizer for the cariogenic bacteria. Similarly, irradiation with the diode laser alone did not cause any significant changes in the viability of planktonic or sessile biofilm cells.

On the other hand, treatment with TBO together with irradiation with the laser resulted in reductions in the viability of *S. mutans* to levels of 13% in planktonic cells and 19% in biofilm cells (Fig. 1). Furthermore, exposure to the laser and TBO caused reductions in the viability of *L. casei* to levels of 30% in planktonic and 13.29% in sessile biofilm cells (Fig. 2). With *A. viscosus* planktonic cells showed a viability of 55% compared to 52% in biofilm cells (Fig. 3). Interestingly, exposure of *S. salivarius* to TBO and laser did not have much effect, with 95% of the planktonic cells remaining viable compared to 86% viability of biofilm cells (Fig. 4).

Two-way analysis of variance (ANOVA) revealed that both the treatment protocol and the species of bacteria as well as the interaction between treatment and species of bacteria had highly significant effects on the viability of the cariogenic bacteria in planktonic and biofilm form ($p < 0.001$). Follow up multi comparison was carried out using Tukey's pairwise comparison to determine any significant differences between the viability of the bacterial species at each treatment protocol as well as the differences between the

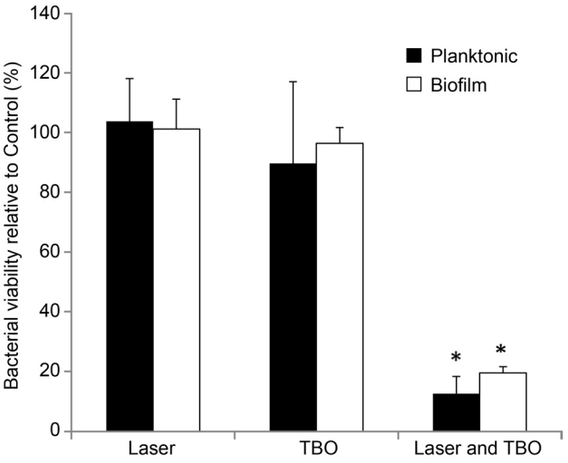


Fig. 1. Viability (mean + SD) of *S. mutans* as planktonic cells in suspension and sessile cells (biofilms) on tooth slices treated with; Laser, TBO, and Laser with TBO relative to controls (100% viability). Asterisk symbol represents statistically significant differences in viability in comparison to treatment with laser alone and TBO alone.

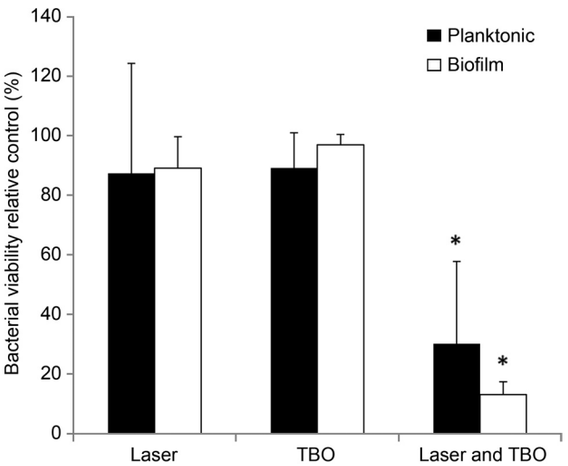


Fig. 2. Viability (mean + SD) of *L. casei* as planktonic cells in suspension and sessile cells (biofilms) on tooth slices treated with; Laser, TBO, and Laser with TBO relative to controls (100% viability). Asterisk symbol represents statistically significant differences in viability in comparison to treatment with laser alone and TBO alone.

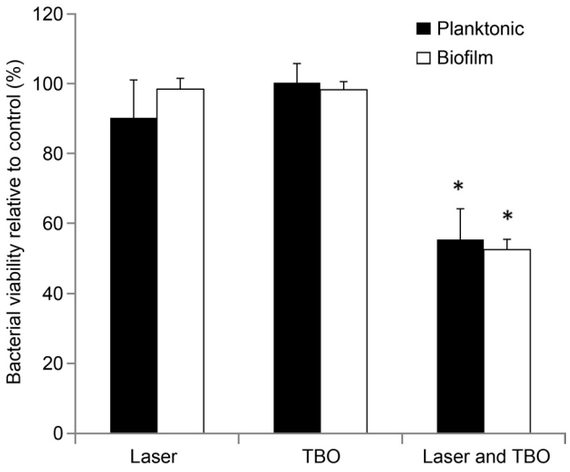


Fig. 3. Viability (mean + SD) of *A. viscosus* as planktonic cells in suspension and sessile cells (biofilms) on tooth slices treated with; Laser, TBO, and Laser with TBO relative to controls (100% viability). Asterisk symbol represents statistically significant differences in viability in comparison to treatment with laser alone and TBO alone.

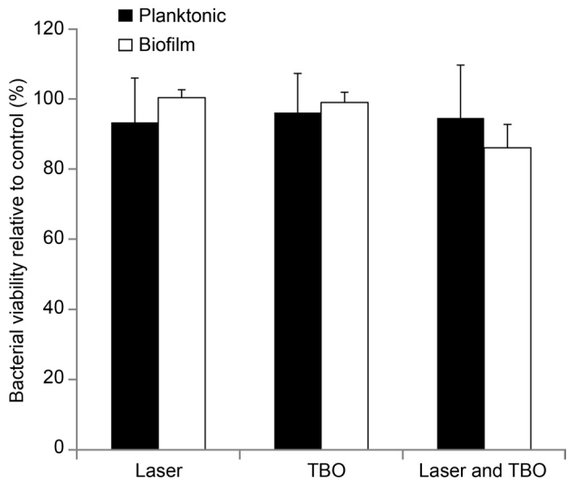


Fig. 4. Viability (mean + SD) of *S. salivarius* as planktonic cells in suspension and sessile cells (biofilms) on tooth slices treated with; Laser, TBO, and Laser with TBO relative to controls (100% viability).

treatment protocols for each bacterial species. When the effects of exposure of planktonic or biofilm cells to laser alone or TBO alone, were examined, no significant reductions in viability of any of the cariogens were observed ($p > 0.05$). However, treatment with TBO and laser caused significant reductions in bacterial viability ($p < 0.05$) in comparison to treatment with laser alone and TBO alone for both the planktonic and biofilm cells for all the bacterial species investigated. Interestingly, Tukey's pairwise comparison showed that differences in viability between planktonic and biofilm cells treated with TBO and laser were not significant ($p > 0.05$).

## Discussion

Various studies have reported that successful lethal photosensitization can be achieved when oral bacteria are grown as planktonic cultures (Burns et al. 1994; Williams et al. 2003; Paulino et al. 2005; Metcalf et al. 2006; Tonon et al. 2015). However, target organisms in oral infections are present within biofilms, which are known to be impervious to the action of many antimicrobial agents, since they are protected by a network of polymeric substances and exhibit differences in structure, metabolism and gene expression (Marsh 2004; Chávez De Paz et al. 2008; Jakubovics et al. 2008; Decker et al. 2014; Van Acker and Coenye 2016).

Different studies have reported that lethal photosensitization of biofilms of cariogenic bacteria could be achieved in root canals and human dentine. However, according to the authors' knowledge, comparative studies of different genera of cariogenic bacteria present as biofilms in the tooth structure are lacking. Thus, the current study compared the sensitivity to photosensitization of three different genera of cariogenic bacteria present as biofilms in coronal tooth slices with that of their planktonic counterparts, to see if there are differences in sensitivity to this mode of therapy. The enamel was removed from the tooth specimens to expose the dentinal area, which is the part of the tooth that the cariogenic bacteria colonize after the enamel surface breaks away in the process of cavity formation. The results indicated a similar level of reduction in the viability of both planktonic and sessile biofilm cells.

Toluidine Blue O was used as the photosensitizer not only because it is very effective in sensitizing bacteria (Williams et al. 2003) but also since its absorption maxima falls within the range of the wavelength of light emitted by the diode (GaAlAs) laser chosen. Exposure of the bacteria to TBO alone had no effects on the viability of planktonic bacteria as well as their biofilm counterparts, in agreement with previous reports (Burns et al. 1994; Wilson et al. 1995; Williams et al. 2003). Furthermore, exposure to laser alone had no effects on the viability of the planktonic or biofilm cells, in contrast to a previous report where exposure of different oral bacteria to light from a diode laser for 1 min resulted in a significant decrease in viability of various oral bacteria (Chan and Lai 2003).

The whole tooth slice specimen was immersed in the culture medium containing the bacteria in order to avoid any variation in the penetration of the bacteria into the dentinal tubules and the tooth slices were incubated with the bacteria for 24 h to allow the formation of a biofilm. The procedure for preparation of the biofilm was in accordance with a previous study (O'Neill et al. 2002).

Biofilms of four species of cariogenic bacteria, (*S. mutans*, *S. salivarius*, *L. casei* and *A. viscosus*), were used since it is well established that they are important in dental caries (Samaranayake et al. 2012). Indeed, *S. mutans* is the initiator of dental caries due to its acidogenic activity that culminates in the degradation of the enamel matrix (Love et al. 2000; van Ruyven et al. 2000). Expansion from this primary focus of enamel degradation results in exposure of the underlying dentine allowing access of microorganisms to the dentinal tubules and subsequently the dental pulp (Love et al. 2000). In succession, lactobacilli play an important role in the progression to a more caries inducing plaque. Lactobacilli are frequently located in the deepest part of the lesion (dentine), under conditions of high acidity for extended periods of time (Munson et al. 2004; Aas et al. 2008). *S. salivarius* is believed to persevere in dental biofilms, colonizing teeth and soft tissues and reported to be intimately involved in health and disease of the oral cavity (Chen et al. 1996; Chen et al. 2000; Gross et al. 2012; Krzyściak et al. 2017). Endogenic Actinomyces constitute between 40 to 80% of the normal flora on adjacent tooth surfaces participating in the aggregation of different species of bacteria during dental biofilm formation and contributing to root caries and periodontal infections (Whittaker et al. 1996; Socransky and Haffajee 1997; Ruby et al. 2002; Do et al. 2017).

The current study found that planktonic cultures of *S. mutans* were the most sensitive (87% killing) followed by *L. casei* (70% killing), *A. viscosus* (45% killing) and finally *S. salivarius* (5% killing). Successful lethal photosensitization of cariogenic bacteria using similar conditions and time of exposure has been previously reported in other studies (Burns et al. 1994; Williams et al. 2003). Indeed, Williams et al. (2003) found 100% killing of *S. mutans* when exposed to lethal photosensitization for 1 min under similar conditions as the current study. Furthermore, Burns et al. (1994) observed appreciable destruction that had been achieved for *S. mutans*, *L. casei* and *A. viscosus* within 30–90 sec exposures to lethal photosensitization. Our results are also in agreement with those of Wilson et al. (1995) who found that when *S. mutans*, *L. casei* and *A. viscosus* were treated by PDT for an exposure time of 1 min, 76% reduction occurred in the viability of *S. mutans* with the same laser but a different sensitizer, while the viability of *A. viscosus* decreased by 37%.

The results also showed that photosensitization of biofilms of cariogenic bacteria in tooth slices resulted in a genus and species-dependent decrease in viability. The most susceptible cariogen was *L. casei* (87% death) followed by *S. mutans* (80 % death), *A. viscosus* (47% death) and *S. salivarius* (14% death). Our results agree with those of Zanin et al. (2006) who reported a slightly greater (95%) reduction in the number of viable *S. mutans* in biofilms cultured on enamel slabs, following PDT with TBO and diode laser. Furthermore, Ricatto et al. (2014) also found significant reductions in the viability of *S. mutans* and *L. casei* using a different sensitizer (Methylene Blue) and a diode laser. In addition, significant reductions in *S. mutans* ($1.08 \pm 1.20$ log) and *Lactobacillus* spp. ($1.69 \pm 1.37$ log) have been observed in dentine from deep carious lesions (Melo et al. 2015).

The results showed that *S. salivarius* was the most resistant strain. There are very few studies, if any, on the effects of PDT on biofilms of *S. salivarius*, according to the authors' knowledge. The reasons behind this resistance to PDT need further investigation.

The current study investigated the effectiveness of antimicrobial PDT on single-species biofilms cultured on coronal tooth slices and future work is in progress to investigate the outcome of PDT on polymicrobial infection encountered in the process of caries formation. The dye/laser combination was found to be effective in achieving significant elimination of biofilms of *L. casei*, *S. mutans* and *A. viscosus* in the tooth structure itself. Surprisingly, *S. salivarius* was comparatively resistant to treatment in both planktonic and sessile state.

Although it remains true that PDT can only be used superficially due to limited light penetration, we believe that PDT will be useful in the case of resin restorations or in fissure sealant application to eliminate any bacteria before applying resin-filling materials. Furthermore, in the case of deep caries with a risk of pulp exposure, the remaining layer that separates the pulp from the cavity can be exposed to PDT to eliminate any bacteria that could be present in this layer or bacteria that have penetrated into the dentinal tubules.

The advantage of PDT is in applying the photosensitizer locally, precisely to the lesion. Subsequent to the administration of the photosensitizer, light of the appropriate wavelength could be conveyed into the intended space specifically using a fiber optic cable. Therefore, with the use of PDT to treat carious lesions perturbation of the normal microbial community at other locations in the oral cavity would not occur (Gross et al. 2012; Lee et al. 2012).

## Conclusion

The results of this in vitro study suggest that sensitization with TBO and exposure to light from a laser diode (GaAlAs) with a wavelength of 670 nm can kill most of *L. casei*, *S. mutans*, *A. viscosus*, and to a lesser extent *S. salivarius*, adhering to coronal tooth slices

and hosted in the dentine. Furthermore, the levels of antimicrobial photosensitization achieved with the cariogenic bacteria hosted in the dentine were similar to that achieved with planktonic cells in suspension.

**Ethical Statement**

Verbal consent was obtained from all patients from whom the extracted teeth samples were obtained. The Institutional Ethics Review Board of Jordan University of Science and Technology approved the consent procedure and the research. The work complied with the World Medical Association Declaration of Helsinki.

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**Conflict of interest**

Authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

# Emodin Reduces the Activity of (1,3)-β-D-glucan Synthase from *Candida albicans* and Does Not Interact with Caspofungin

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Abstract

Candidiasis is the most common opportunistic yeast infection, with *Candida albicans* as a paramount causative species. (1,3)-β-D-glucan is one of the three main targets of clinically available antifungal agents used to treat *Candida* infections. It is one of the most abundant fungal cell wall components. Echinocandins represent the newest class of antifungals affecting cell wall biosynthesis through non-competitive inhibition of (1,3)-β-D-glucan synthase. Therefore, treatment with echinocandins causes defects in fungal cell integrity. In the present study, similar activity of emodin (6-methyl-1,3,8-trihydroxyanthraquinone) has been revealed. Many reports have already shown the antifungal potential of this pleiotropic molecule, including its activity against *C. albicans*. The aim of this report was to evaluate the activity of emodin towards a new molecular target, i.e. (1,3)-β-D-glucan synthase isolated from *Candida* cells. Moreover, given the identical mechanism of the activity of both molecules, interaction of emodin with caspofungin was determined. The study revealed that emodin reduced (1,3)-β-D-glucan synthase activity and increased cell wall damage, which was evidenced by both a sorbitol protection assay and an aniline blue staining assay. Furthermore, the synergy testing method showed mainly independence of the action of both tested antifungal agents, i.e. emodin and caspofungin used in combination.

Key words: *Candida albicans*, caspofungin, echinocandins, emodin, (1,3)-β-D-glucan synthase

## Introduction

Candidiasis is one of the most prevalent superficial and deep-seated fungal infections in humans and, as such, a major global health problem, which is additionally associated with a high mortality rate. The most pervasive and problematic cause of infections of all *Candida* species is *Candida albicans* – a part of the commensal microbiota of more than half of the healthy population. It is a cause of both opportunistic and invasive fungal infections (Pfaller and Diekema 2007; Sardi et al. 2013). Yeast infections frequently develop in immunocompromised patients with AIDS, cancer, and neutropenia as well as those receiving immunesuppressive and antibiotic therapy (Canela et al. 2018). Recent reports indicate that *Candida* infections are often associated with bipolar disorder and schizophrenia (Severance et al. 2016). The pathogenicity of *Candida* species is supported by a wide range of virulence factors and fitness attributes, such as biofilm formation, polymorphism, thigmotropism, phenotypic switching, secretion of hydrolytic enzymes, quick adaptation to fluctuations in environmental pH, metabolic flexibility, and strong stress response mechanisms (Mayer et al. 2013; Martins et al. 2014).

Due to the similarity of human and fungal cells, discovery of selective antifungal drugs is extremely difficult. Nevertheless, there are some elements differentiating both types of cells. One of them is the cell wall that does not exist in mammalian cells. (1,3)-β-D-glucan is the main polysaccharide in the fungal cell wall. It is synthesized in the fungal cell by glucan synthase located in the cell membrane. This enzyme is regarded as a molecular target in the search for compounds with potential antifungal activity. Echinocandins, the current antifungal drugs are the inhibitors of (1,3)-β-D-glucan synthase (Denning 2003).

Echinocandins represented by anidulafungin, caspofungin, and micafungin target the synthesis of (1,3)-β-D-glucan polymers through non-competitive

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inhibition of the glucan synthase enzyme. Inhibition of a fungal-specific target by these antibiotics leads to defects in fungal cell wall integrity (Pianalto and Alspaugh 2016).

Despite the effectiveness of echinocandins in the control of many fungal infections, antifungal resistance and defensive mechanisms resulting from the use of these drugs have been observed in fungal cells. One of the beneficial alternatives for potentiating the antifungal drugs is combination therapy comprising an antibiotic with a natural product (Zacchino et al. 2017). A combination of several medicinal substances can significantly improve the therapeutic properties and reduce the effective concentration of antibiotics while eliminating their side effects (Martins et al. 2014; Singh and Yeh 2017).

In the light of these facts, the medicinal potential of phytochemicals, their synergistic action with antifungal agents, and their interrelated mechanisms of action have been extensively studied (Kanafani and Perfect 2008; Sher 2009; Agarwal et al. 2010). Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) is one of the most promising natural compounds. Many reports regard emodin as a plant component with antioxidant, antibacterial, antiviral, antimutagenic, antitumor, and immunosuppressive properties (Shrimali et al. 2013; Dong et al. 2016). The latest research on the biological activity of this compound points to its anti-*C. albicans* activity (Kong et al. 2009; Janeczko et al. 2017).

The aim of the present study was to evaluate the inhibitory properties of emodin against (1,3)-*β*-D-glucan synthase from *C. albicans* cells. Moreover, the influence of the compound on *C. albicans* cell wall destruction was evidenced with the use of a sorbitol protection assay and aniline blue staining. A checkerboard microliter plate assay was also used to determine the FICI (Fractional Inhibitory Concentration Index) in order to evaluate the combined activities of emodin and caspofungin against *C. albicans* strains by determining the FICI (Fractional Inhibitory Concentration Index).

## Experimental

### Materials and Methods

***C. albicans* strains.** The experiments were performed on *C. albicans* reference strain ATCC 10231. Additionally, 20 clinical strains isolated from urinary tracts and 20 clinical strains isolated from reproductive systems of gynecological patients of Jan Boży Independent Public Provincial Hospital in Lublin, Poland, were included in this study. The strains were identified using VITEK 2 YST IC CARDS (Biomerieux).

**Determination of Minimal Inhibitory Concentrations (MICs)**. The MICs of emodin (Sigma-Aldrich, USA), caspofungin (Sigma-Aldrich, USA), and amphotericin B (Sigma-Aldrich, USA) were determined with the broth dilution method as recommended by CLSI, with some modifications (CLSI 2017). Two-fold serial dilutions of emodin (0.8–400 μg/ml) or antibiotics (0.015–10 μg/ml) were prepared in 96-well microtiter plates using RPMI-1640 medium (with L-glutamine and phenol red, without bicarbonate) (Sigma-Aldrich, USA) buffered with 0.165 M 3-(N-morpholino)propane sulfonic acid (MOPS) (Sigma-Aldrich, USA). Adjacent wells of the microtiter plates contained 100 μl of each dilution. The inoculum was prepared by dilution of *C. albicans* cells with RPMI. The turbidity of this suspension was adjusted to $1–5 \times 10^3$ at a 530 nm wavelength. After addition of 20 μl of the inoculum to the wells, the plates were incubated at 37°C for 48 hours. 100 μl of uninoculated medium was included as a sterility control (blank). The MIC was taken as the lowest concentration of disinfectant that inhibits fungal growth. The experiments were performed in triplicate.

**Sorbitol protection assay.** The sorbitol protection assay was carried out to determine the effect of emodin on the destabilization of the fungal cell membrane. To this end, duplicate plates containing either emodin or amphotericin B and caspofungin were prepared as controls. One plate from each pair contained only the substance tested and the other plate contained an adequate antifungal and, additionally, 0.8 M sorbitol as an osmotic protectant (Frost et al. 1995). MICs for each trial were determined by the modified CLSI protocol as described above. Each assay was performed in triplicate.

**Preparation and quantification of (1,3)-*β*-D-glucan synthase.** (1,3)-*β*-D-glucan synthase from *C. albicans* cells (ATCC 10231) was prepared using the method proposed by Shedletzky et al. (1997) with some modifications described by Lee and Kim (2016). The enzyme was isolated from *C. albicans* cells cultivated in 1 l of Sabouraud Dextrose Agar Broth (Biocorp, Poland) at 37°C for 16 h. The cells were homogenized in a Bead Beater (Minilys Homogenizer, Bertin Instruments) in 12 cycles of 1 min with 0.5-mm acid-washed glass beads. The protein concentration in the microsome and membrane fraction was measured using the Bradford method in accordance with the manufacturer's instructions (Sigma-Aldrich, USA). The (1,3)-*β*-D-glucan synthase assay was performed according to the method developed by Frost et al. (1995) and modified by Lee and Kim (2016). The glucans stained specifically with aniline blue solution (0.1%) were a measure of the enzyme activity. Fluorescence was measured using a spectrofluorometer (Pharmacia Biotech) at 400-nm excitation and 460-nm emission wavelengths. The effect of emodin on the enzyme activity was determined at concentrations corresponding to the MIC/4, MIC/2, MIC, and 2 × MIC, and DMSO was used as a control.

The assays were performed in triplicate in three independent experiments.

**Aniline blue staining of (1,3)-$\beta$-glucan in the *C. albicans* cell wall.** The aniline blue staining method and fluorescence microscopy were used to visualize the effect of emodin and caspofungin on (1,3)-$\beta$-D-glucans in the *C. albicans* (ATCC 10231) cell walls. The yeast cells at the exponential phase were harvested by centrifugation at 4500 × g at 4°C for 5 min. Next, the cells were washed twice and resuspended in 0.85% NaCl. Emodin at concentrations corresponding to MIC/2 and MIC/4, caspofungin at MIC/2, and 1% DMSO as control were added to the cell suspensions and incubated at 37°C for 10 h. The cells were harvested and washed in 0.85% NaCl; next, the cell density of each experimental group was adjusted to $1 \times 10^8$ cells/ml and the cells were resuspended in an aniline blue solution (0.1%). The samples were stained at 50°C for 30 min. A drop of each suspension was squashed between the microscope slide and the cover glass. The preparation was sealed and examined in a fluorescence microscope under UV illumination (Nicon). Images were taken with a cooled monochrome camera.

**Caspofungin – emodin combination assay (a checkerboard method).** Interactions between caspofungin and emodin were measured by calculation of the fractional inhibitory concentration index (FICI). A total of l00 µl of RPMI-1640 medium was distributed into each well of the microdilution plates. The first antibiotic of the combination – caspofungin was serially diluted along the ordinate at a concentration range of 0–1.2 µg/ml, while the other drug – emodin was diluted along the abscissa at a concentration range of 0–100 µg/ml. The inoculum was prepared from *C. albicans* in RPMI-1640 medium as described in the MIC assay. Each microtiter well was inoculated with 20 µl of the yeast inoculum and the plates were incubated at 37°C for 48 h. The MIC values were detected with the naked eye. The FICI values were calculated for each well with the equation FICI = FICA + FICB = (MICA+B/MICA) + (MICB + + A/MICB), where MICA and MICB are the MICs of drugs A and B alone, respectively, and MICA + B and MICB + A are the concentrations of the drugs applied in combination, respectively, in all the wells corresponding to the MIC. A combination of two drugs is considered synergistic when the FICI is ≤ 0.5, indifferent when the FICI is > 0.5 to ≤ 4, and antagonistic when the FICI is > 4 (Odss 2003; Petersen et al. 2006).

## Results and Discussion

Emodin is a natural anthraquinone derivative found mainly in the roots and rhizomes of numerous plants. Pharmacological studies have demonstrated that emodin with its various biological functions has been used in the treatment of cancers and inflammatory diseases (Wei et al. 2013; Dong et al. 2016; Monisha et al. 2016). The unique therapeutic potential of emodin results from its ability to interact with many molecular targets, e.g. protein kinases, NADH-oxidase, topoisomerase II, survivin, XIAP, STAT3, p53, and p21 (Shrimali et al. 2013). Furthermore, emodin was found to have antimicrobial activity (Alves et al. 2004; Kong et al. 2009; Liu et al. 2013; Cao et al. 2015; Liu et al. 2015; Janeczko et al. 2017).

In this study, the antifungal activity of emodin against the reference and clinical strains of *C. albicans* has been confirmed by the CLSI method in RPMI medium. The minimal inhibitory concentration against the standard strain was 12.5 µg/ml. The control antibiotics, caspofungin and amphotericin B, inhibited yeast growth at concentrations of 0.15 µg/ml and 1 µg/ml, respectively (Table I). Moreover, emodin suppressed the growth of all clinical strains isolated from the urinary tracts or the vaginas of the gynecological patients. The activity against these species has been shown at values of MICs between 6.25 and 50 µg/ml. Also, all isolates were susceptible to caspofungin. The MICs ranged from 0.03 to 0.6 µg/ml. The MIC values of the antifungal agents tested individually are summarized in Table II and Table III. These results were comparable to MICs obtained in our previous work (Janeczko et al. 2017). As demonstrated in the previous study, emodin suppressed the growth of *C. albicans* and other reference strains, such as *C. krusei*, *C. parapsilosis*, and *C. tropicalis*, as well as clinical *Candida* strains. In addition, fungicidal activity against these species has been shown at values of MICs and MFCs (Minimal Fungicidal Concentrations) between 12.5 and 200 µg/ml. Moreover, we have proved that this compound has anti-virulent potential by reducing hyphal formation, suppressing adhesion, which is the first and critical phase of fungal infection, and destabilizing fully established biofilm. In terms of the high pleiotropic nature of emodin, it has been confirmed that this compound is an effective inhibitor of protein kinase 2 (CK2) isolated from *C. albicans* cells (Janeczko et al. 2017).

In order to verify the influence of emodin on *C. albicans* cells, the previous research on the molecular

Table I
Effect of sorbitol on the MICs of emodin and antibiotics against *C. albicans* ATCC 10231.

| | MIC (µg/ml) | |
|---|---|---|
| | Without sorbitol | With sorbitol |
| Emodin | 12.5 | 25 |
| Caspofungin | 0.15 | 0.6 |
| Amphotericin B | 1 | 1 |

Table II
*In vitro* activity of emodin and caspofungin alone and in combination assessed by the broth dilution assay against clinical strains of *C. albicans* isolated from urinary tracts.

| Strain No. | MIC of the drug used alone (μg/ml) | | MIC of the drug used in combination (μg/ml) | FICI | Interaction |
|---|---|---|---|---|---|
| | Emodin | Caspofungin | Emodin + Caspofungin | | |
| 1 | 12.5 | 0.15 | 12.5 + 0.3 | 3 | Indifferent |
| 2 | 12.5 | 0.07 | 12.5 + 0.3 | 5.28 | Antagonism |
| 3 | 25 | 0.3 | 25 + 0.6 | 3 | Indifferent |
| 4 | 25 | 0.3 | 25 + 0.3 | 2 | Indifferent |
| 5 | 50 | 0.6 | 50 + 1.2 | 3 | Indifferent |
| 6 | 50 | 0.15 | 50 + 0.3 | 3 | Indifferent |
| 7 | 12.5 | 0.15 | 12.5 + 0.3 | 3 | Indifferent |
| 8 | 12.5 | 0.07 | 12.5 + 0.3 | 5.28 | Antagonism |
| 9 | 25 | 0.03 | 25 + 0.15 | 6 | Antagonism |
| 10 | 25 | 0.3 | 25 + 0.15 | 1.5 | Indifferent |
| 11 | 25 | 0.15 | 25 + 0.3 | 3 | Indifferent |
| 12 | 50 | 0.6 | 50 + 0.3 | 1.5 | Indifferent |
| 13 | 50 | 0.3 | 50 + 0.3 | 2 | Indifferent |
| 14 | 50 | 0.6 | 50 + 0.6 | 2 | Indifferent |
| 15 | 25 | 0.3 | 25 + 0.15 | 1.5 | Indifferent |
| 16 | 6.25 | 0.03 | 12.5 + 0.06 | 4 | Indifferent |
| 17 | 50 | 0.6 | 50 + 0.6 | 2 | Indifferent |
| 18 | 50 | 0.3 | 50 + 0.3 | 2 | Indifferent |
| 19 | 50 | 0.15 | 25 + 0.15 | 1.5 | Indifferent |
| 20 | 25 | 0.6 | 50 + 0.6 | 3 | Indifferent |

impact of this substance has been extended to examination of its effect on cell wall damage. The yeast cell wall serves many functions, inter alia providing cell rigidity and shape, metabolism, ion exchange, and interactions with host defense mechanisms. Since the cell wall is not present in mammalian cells, it is an excellent target for specific antifungal antibiotics with higher selectivity towards pathogen cells and lower toxicity against host cells (Denning and Hope 2010). The damaging effects on the *C. albicans* cell wall were evaluated on the basis of the MICs of emodin, caspofungin (positive control), and amphotericin B (negative control) in the absence and presence of sorbitol. Reduction of crucial cell wall components by antifungal agents will lyse cells in the absence of an osmoprotectant. These effects can be recovered in the presence of such osmoprotectants as sorbitol: in this case, cells will continue to grow. As shown in Table I, the MIC of emodin increased twice in relation to the sample without any osmoprotectant after 2 days of incubation. The MIC of caspofungin increased three times without any sorbitol after the same incubation time. In contrast, the MIC of amphotericin B did not change. The increase in the MIC in the presence of sorbitol demonstrated that emodin was involved in cell wall synthesis.

The fungal cell wall is a unique structure built of $\alpha$- and $\beta$-linked glucans, chitin, polysaccharides, and mucopolysaccharides. Many of these biopolymers are essential for proper functioning of fungal cells. Enzymes synthesizing these biopolymers could be desirable antifungal targets (Wiederhold 2018). Since emodin is a highly pleiotropic molecule capable of interacting with several major molecular targets and damaging *C. albicans* cell walls, the influence of the compound on the activity of (1,3)-$\beta$-D-glucan synthase (GS) has been analyzed. This enzyme is a glucosyltransferase involved in synthesis of 1,3-$\beta$-D-glucan in fungi – one of the main molecular targets used in clinically available antifungals and also a pharmacological target for echinocandins (Denning 2003). Inhibition of GS activity and the following depletion of $\beta$-glucans from the fungal cell wall result in cell lysis under osmotic stress (Frost et al. 1995).

An aniline blue assay was used to determine the effect of emodin on the activity of (1,3)-$\beta$-D-glucan synthase obtained from a microsomal membrane fraction from *C. albicans*. The decrease in the GS activity after the treatment with the anthraquinone tested was shown as a percentage of the DMSO control. As shown in Fig. 1, emodin reduced the GS activity approxi-

Table III
*In vitro* activity of emodin and caspofungin alone and in combination assessed by the broth dilution method against clinical strains of *C. albicans* isolated from vaginas.

| Strain No. | MIC of the drug used alone (μg/ml) | | MIC of the drug used in combination (μg/ml) | FICI | Interaction |
|---|---|---|---|---|---|
| | Emodin | Caspofungin | Emodin + Caspofungin | | |
| 1 | 50 | 0.3 | 50 + 0.3 | 2 | Indifferent |
| 2 | 50 | 0.6 | 50 + 0.6 | 2 | Indifferent |
| 3 | 25 | 0.15 | 25 + 0.3 | 3 | Indifferent |
| 4 | 50 | 0.3 | 50 + 0.3 | 2 | Indifferent |
| 5 | 25 | 0.15 | 25 + 0.3 | 3 | Indifferent |
| 6 | 50 | 0.07 | 25 + 0.15 | 2.64 | Indifferent |
| 7 | 50 | 0.3 | 50 + 0.6 | 3 | Indifferent |
| 8 | 50 | 0.6 | 25 + 0.3 | 1 | Indifferent |
| 9 | 12.5 | 0.3 | 25 + 0.3 | 3 | Indifferent |
| 10 | 50 | 0.15 | 50 + 0.6 | 5 | Antagonism |
| 11 | 50 | 0.07 | 50 + 0.15 | 3 | Indifferent |
| 12 | 50 | 0.6 | 50 + 0.3 | 1.5 | Indifferent |
| 13 | 25 | 0.3 | 50 + 0.3 | 3 | Indifferent |
| 14 | 25 | 0.6 | 50 + 0.15 | 2.25 | Indifferent |
| 15 | 25 | 0.3 | 50 + 0.15 | 2.5 | Indifferent |
| 16 | 12.5 | 0.03 | 25 + 0.15 | 5 | Antagonism |
| 17 | 12.5 | 0.6 | 12.5 + 0.6 | 2 | Indifferent |
| 18 | 50 | 0.3 | 50 + 0.6 | 3 | Indifferent |
| 19 | 50 | 0.15 | 25 + 0.07 | 1 | Indifferent |
| 20 | 50 | 0.6 | 50 + 0.3 | 1.5 | Indifferent |

mately to 67.6% ± 3.4%, 76% ± 4%, 87.4% ± 5,2%, and 91.2% ± 3,8% at concentrations equal to 2 × MIC, MIC, MIC/2, and MIC/4, respectively, when compared to the DMSO control cells. The reduced activity of GS proved that emodin prevented the synthesis of the *C. albicans* cell wall, and therefore, the effect of its activity corresponds to that of echinocandins.

Additionally, aniline blue was used to verify the *in vivo* influence of emodin on changes in the content of (1,3)-*β*-D-glucans in *C. albicans* by inhibition of their

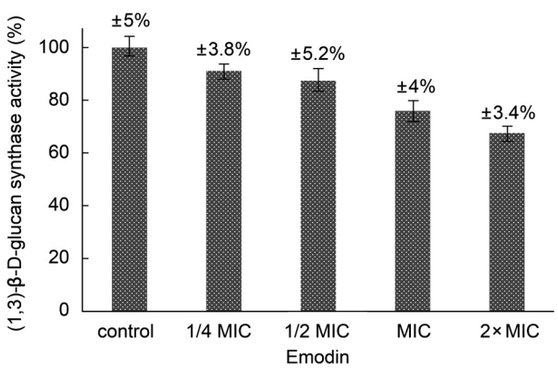


Fig. 1. Effect of emodin on (1,3)- *β*-D-glucan synthase activity.

biosynthesis. This fluorescent dye binds to (1,3)-*β*-D-glucans in the cell wall. The biosynthesis of *C. albicans* cell wall components was shut down by inhibition of GS. The fungi were grown in the presence of emodin at the concentrations of 6.25 μg/ml and 3.12 μg/ml corresponding to MIC/2 and MIC/4, respectively or in the presence of caspofungin at 0.07 μg/ml (MIC/2). The control contained DMSO at the same concentration as in the samples with emodin. As shown in Fig. 2, the intensity of fluorescence of fungal cells stained with blue aniline was lower in cells treated with emodin and caspofungin than in the controls. The apparent significant loss of fluorescence of the fungal cell walls and, above all, the disintegration of cells under the pressure of caspofungin at MIC/2 correlated with the sensitivity of this species to echinocandin, i.e. a 1,3-*β*-D-glucan synthesis inhibitor (Fig. 2B). The treatment of *C. albicans* cells with emodin at concentrations MIC/2 and MIC/4 resulted in reduction of the number of cells, but the reduction of the glucan content in the cell walls was almost imperceptible (Fig. 2C and Fig. 2D).

Recently, natural product screening has also been a source of a number of distinct GS inhibitors. Antifungal activity was shown by natural lipopeptides and triterpenes containing a polar (acidic) moiety (Vicente

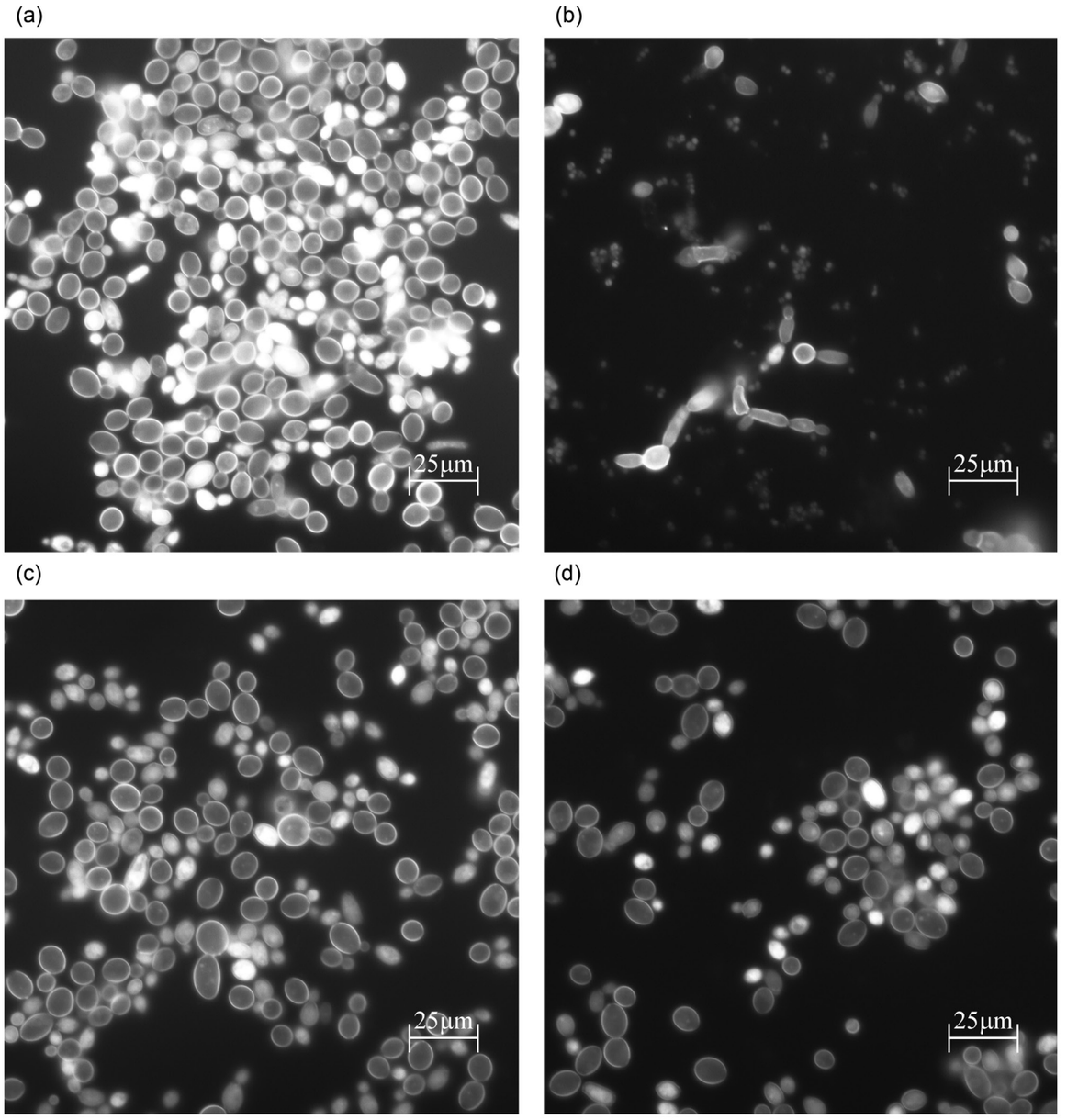


Fig. 2. Aniline blue staining of *C. albicans* cell walls. A) treatment with DMSO at 1% (control); B) caspofungin at 0.07 µg/ml; C) emodin at 6.25 µg/ml; D) emodin at 3.12 µg/ml.

et al. 2003). In addition, several new investigational agents are currently under development. Among these, there are semi-synthetic enfumafungins modified by replacement with amino ethers (Apgar et al. 2015), SCY-078, which derives from enfumafungin, as well and a cyclic hexapeptide rezafungin (CD101, biafungin, previously SP3025) (Wiederhold 2018).

The clinical success of the echinocandins is associated with their fewer toxic side effects in comparison to polyenes and their fewer drug-drug interactions compared to azoles. These drugs are primarily used for the treatment of invasive candidiasis and as an alternative therapy for aspergillosis treatment (Odds et al. 2003; Denning and Hope 2010). Unfortunately, the effectiveness of these antibiotics is compromised due to a critical increase in the emergence of drug-resistant *Candida* strains. In the face of this problem, another strategy has been developed to overcome the treatment failures by combining different antifungals. Many reviews indicate that the combination of antibiotics, phytochemicals, or both natural plant products and well-known antibiotics offers significant potential for the development of novel antimicrobial therapies and treatment of several diseases caused by microorganisms. The advantages of the synergistic action of antibiotics and plant extracts include reduction of

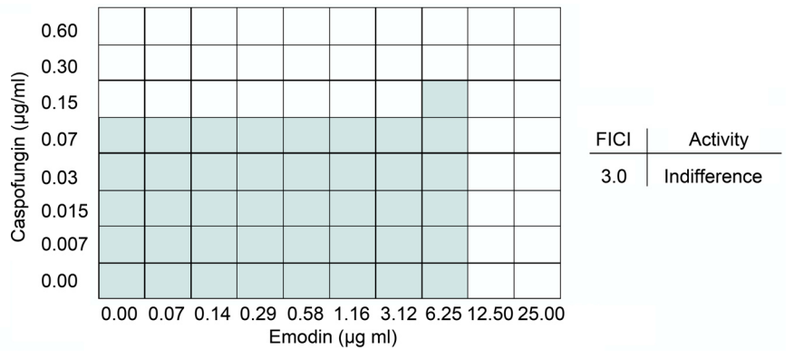


Fig. 3. The checkerboard method showing unresponsiveness of the emodin-caspofungin combination. The resulting checkerboard included each combination of emodin and caspofungin, with wells containing the highest concentration of each compounds in opposite corners (darkened fields mean cell growth; light fields mean no growth).

undesirable effects and increased efficiency. It is also important to increase the stability and bioavailability of free agents and achieve an adequate therapeutic effect with relatively small doses compared to any synthetic medication (Hemaiswarya et al. 2008).

Since emodin affects the activity of (1,3)-$\beta$-D-glucan synthase in the same range as caspofungin, the interactions between this anthraquinone and the antibiotic were investigated. In this case, two possibilities of interaction could be expected – synergism or antagonism in the action against *C. albicans* cells. The third option was indifferent interaction between the two tested substances. Synergism is defined as a positive interaction occurring when two agents combined together exert an inhibitory effect that is greater than the sum of their individual effects. In turn, the term antagonism is used when the effect of both drugs together is worse than the effect of either alone. Then, indifference means that no effect is exhibited. The caspofungin-emodin combination effect was measured using the checkerboard microtiter plate method and calculation of the fractional inhibitory concentration index (FICI). The course and result of the experiment for the reference *C. albicans* strain is schematically shown in Fig. 3. The combination of both antifungals showed a tendency towards indifference between the tested compounds at most concentrations and ratios. Thus, emodin at 0.19–3.12 µg/ml did not affect the caspofungin activity against *C. albicans*. Similarly, caspofungin at 0.007–0.6 µg/ml did not change the MIC values for emodin. Only in one case, the MIC of caspofungin increased from 0.15 µg/ml to 0.3 µg/ml in the presence of 6.25 µg/ml emodin. Based on the MIC values in various concentration combinations of both antifungal compounds, the FICI was 3 and did not show any interactions between the compounds.

The checkerboard assays evaluated against 40 clinical isolates of *C. albicans* showed that the combination of emodin with caspofungin changed mainly the MIC values with respect to caspofungin. MICs increased for 18 strains, decreased for nine strains, and remained unchanged for 13 isolates. The composite emodin/caspofungin caused a change in the MICs with respect to emodin to a lesser extent. The MIC values decreased for four strains and increased for five strains. They were ca. 2–4-fold lower or higher than the values for the compounds applied alone. The FICI values of the combinations of the antifungal drugs ranged from 1 to 6. This combination showed predominantly indifferent interactions between emodin and caspofungin (87.5% isolates) with the FICI in the range from 1 to 4. An antagonistic effect was proved only against five strains tested (12.5%) with FICI > 4. Otherwise, no synergism was observed (FICI < 0.5). These results were comparable to the FICI of the *C. albicans* reference strain. The MIC and FICI values of the antifungal agents tested in combination are summarized in Table II and Table III.

In conclusion, as shown above, the antifungal activity of emodin against *C. albicans* may be related to the inhibition of (1,3)-$\beta$-D-glucan synthase activity, leading to disruption of (1,3)-$\beta$-D-glucans in the fungal cell wall. This completely new molecular target for emodin is highly desirable due to the high specificity of this type of antifungals in relation to host cells. The novel mechanism of emodin action could hypothetically amplify the activity of echinocandins; however, in combination with caspofungin, this anthraquinone shows indifferent or antagonistic interactions. The data from the studies of the interactions between emodin/caspofungin suggest that these combinations could not be an effective strategy against *C. albicans* infections.

**Conflict of interest**

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

# Primary and Secondary Bacteremia Caused by *Proteus* spp.: Epidemiology, Strains Susceptibility and Biofilm Formation

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Abstract

*Proteus* spp. is an etiological factor of urinary tract and bloodstream infections. The aim of this study was the retrospective analysis of susceptibility of *Proteus* spp. strains isolated from bloodstream infections (BSIs) as well as similarity evaluation of the strains isolated from different clinical samples. *Proteus* spp. strains were isolated in 2009–2017 from hospital patients. Identification was based on the colony's morphology and biochemical or MALDI-TOF MS analyzes. The antibiotic susceptibility test was done using the diffusion method. Biofilm formation was evaluated with microplate method using TTC. Bacteremia caused by *Proteus* spp. was found in 97 patients, mainly secondary to urinary tract infection. Most of the strains were susceptible to piperacillin with tazobactam (95.9%) and amikacin (86.7%). Elderly patients have a higher risk of mortality after BSIs caused by *Proteus* spp. A detailed analysis was made for randomly chosen 26 strains isolated from 11 patients with *Proteus mirabilis* bacteremia. Using PFGE, we found that 10 (90.9%) isolates, collected from different clinical specimens of the same patient, were genetically identical.

Key words: antimicrobial susceptibility, bacteremia, biofilm, *Proteus* spp.

## Introduction

*Proteus* spp. is widely spread in the environment. It may occur in water, soil, manure or sewage, and it is also a component of human and animal gastrointestinal tract microbiome (O'Hara et al. 2000; Drzewiecka 2016). In the current taxonomy of *Proteus* genus, eight species are included: *Proteus mirabilis*, *Proteus vulgaris*, *Proteus penneri*, *Proteus hauseri*, *Proteus cibarius*, *Proteus columbae*, *Proteus incostans*, and *Proteus terrae*. *P. mirabilis* is the most common species of this genus isolated from human infections (Różalski et al. 2007; Armbruster and Mobley 2012).

In addition to be a leading cause of urinary tract infections (UTI) and chronic wound infections (Endimiani et al. 2005), *P. mirabilis* can also cause bloodstream infections. *P. mirabilis* is found in 1–3% of all BSIs (Tumbarello et al. 2012). Other *Proteus* species are rare in BSIs and occur at rates of less than 0.1 per 100 000 (Laupland et al. 2014).

The most common source of bacteria in BSIs caused by *P. mirabilis* is the urinary tract (UT) (Watanakunakorn and Perni 1994). According to Watanakunakorn and Perni (1994) and Kim et al. (2003), UT is a source of 47.6–52.8% BSIs caused by *P. mirabilis* (Watanakunakorn and Perni 1994; Kim et al. 2003).

Among infectious diseases, BSIs are a major cause of mortality worldwide (Laupland and Church 2014). Endimiani et al. (2005) found that mortality rate attributable to *P. mirabilis* BSIs is higher in BSIs cases caused by strains with extended spectrum beta-lactamases (ESBL). The presence of beta-lactamases may seriously limit BSIs treatment options. *Proteus* spp. is inherently resistant to polymyxins that are used, beside carbapenems, in empiric treatments of BSIs caused by Gram-negative bacilli. *P. mirabilis* might produce likewise, the AmpC-type cephalosporinases and carbapenemases (KPC) (Wang et al. 2014; Di Pilato et al. 2016).

The aims of this study were: a) retrospective susceptibility analysis of *Proteus* spp. strains isolated from BSIs, b) the similarity evaluation of 26 *P. mirabilis* strains isolated from clinical samples collected from randomly chosen 11 hospital patients.

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## Experimental

### Materials and Methods

**Identification and susceptibility testing of clinical *Proteus* spp. strains.** *Proteus* spp. strains were isolated from 2009 to 2017 from patients of the Antoni Jurasz University Hospital No 1 in Bydgoszcz, Poland. Basic information, i.e., age, sex of patients, and the samples collected were determined using laboratory data. Details, i.e.: short-term (14 days) mortality after bacteremia, length of stay, period from admission to the hospital to the onset of *Proteus* spp. bacteremia, presence of urinal or central venal catheters, the previous surgery, and additional diseases were determined by using electronic administrative data. We did not find any detailed information about 15 patients hospitalized in 2009–2010; they were excluded from epidemiological analyzes.

The strains were identified at the Department of Microbiology: a) between 2009 and 2011 the identification was based on colony's morphology and the results of biochemical reactions (API 32E, BioMériuex), b) between 2011 and 2014 – on biochemical reaction results included in the VITEK GN cards (BioMériuex), and c) since 2014 – by using MALDI-TOF MS technique (Microflex, Bruker).

Susceptibility of *Proteus* spp. strains was examined: in 2009 and 2010 by using the disk diffusion method and interpreted in accordance with the Clinical and Laboratory Standard Institute (CLSI 2009) recommendation; since 2011 – using VITEK AST cards (BioMériuex) and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2016). The meropenem susceptibility was evaluated since 2016.

Extended-spectrum beta-lactamases (ESBLs) were examined according to the CLSI recommendation by using the double disk synergic test (DDST).

Primary bacteremia was defined as the bacteremia for which no source of infection was documented. Bacteremia was defined as secondary when laboratory examination showed infection by the same microorganism at a distant site in the same time or up to three days earlier.

Until the investigation, the selected strains were stored in BHI with 20.0% glycerol at 70°C.

**Genetic similarity and phenotypic characterization of *P. mirabilis* strains.** A detailed analysis was performed for 26 strains isolated from 11 (A-K) randomly chosen patients with *P. mirabilis* bacteremia. Strains were isolated from three different samples from four patients, and from two different samples – from seven patients. Two patients had urethral catheters, and one – peripheral venous catheter.

**Susceptibility patterns.** The susceptibility assays of *P. mirabilis* strains were done using the diffusion method according to CLSI recommendation, including the following antibiotics in discs (Emapol): trimethoprim TRM (5 μg), norfloxacin NOR (10 μg), netilmicin NET (30 μg), tobramicin TOB (10 μg), ticarcilin TIC (75 μg), amikacin AMK (30 μg), imipenem IMP (10 μg), ciprofloxacin CIP (5 μg), cephoperazone CEP (75 μg), piperacillin PIP (100 μg), ampicillin AM (10 μg), ticarcillin with clavulanic acid TIM (85 μg), cefuroxime CXM (30 μg), ampicillin with sulbactam UNA (20 μg), gentamicin GEN (10 μg). All results were interpreted according to EUCAST (2016).

**Biofilm formation.** Biofilm formation by 26 *P. mirabilis* strains was evaluated based on the method described previously by Kwiecińska-Piróg et al. (2013a). The method is based on the measurement of absorbance of formazan (dissolution product of 2,3,5-triphenyl-tetrazolium chloride, TTC; Avantor; $\lambda = 470$ nm) (BioTek; Synergy HT Multi-detection; KC4 program). The assay was done in three independent repetitions. Two reference strains from the American Type Culture Collection (ATCC): *Staphylococcus aureus* ATCC 6538 (strong biofilm former) and *Escherichia coli* ATCC 35218 (weak biofilm former) were used as the positive and negative control, respectively.

The A value was calculated based on the average (x) and threefold standard deviation (SD) of blank absorbance. The strains which had an absorbance above the A value were classified as being able to biofilm formation. All strains with an absorbance in the range between A and $2 \times A$ were classified as weak biofilm formers (W), in the range between $2 \times A$ and $4 \times A$ – as moderate biofilm formers (M), and above $4 \times A$ – as strong biofilm formers (S).

**Dienes phenomenon testing.** To examine the Dienes phenomenon of *P. mirabilis* strains, single colonies, previously cultured on MacConkey Agar, were inoculated as points on blood agar plates (Oxoid). The strains isolated from the same patient were inoculated on one plate. Plates were incubated at 37°C. After 24 hours, demarcation lines between strains were observed. When strains were non-identical, colonies were separated by the demarcation line (0.5–2 mm wide) (Fig. 1A). When strains were identical – there was any demarcation line (Sabbuda et al. 2003) (Fig. 1B).

**Genetic similarity of *P. mirabilis* strains.** Genetic similarity of *Proteus* spp. strains was evaluated by pulsed field gel electrophoresis (PFGE) using *Sfi*I (Fermentas) restriction enzyme cleavage, as previously described by Sabbuba et al. (2003) with own modifications, described by Kwiecińska-Piróg et al. (2013b). Comparative analysis was performed using the Quantitative One software according to Dice.

**Statistical analysis.** Statistical analysis was performed using Statistica 12.5 PL (StatSoft) software. The confidence interval for all tests was $\alpha = 0.05$. To compare variables, the $chi^2$ test with Yates' correction was used, if necessary.

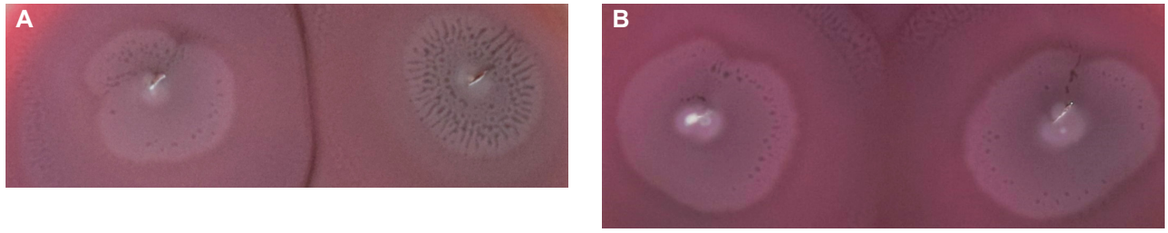



Fig. 1. The Dienes phenomenon: A) non-identical strains, B) identical strains.

## Results

***Proteus* spp. blood stream infections.** In the examined time period (2009–2017), BSIs were found in 3956 cases in 2829 patients. BSIs caused by *Proteus* spp. were confirmed in 98 cases (2.5%) in 97 patients (3.4%). In one female patient, secondary urinary tract bacteremia was recognized two times with a six-months interval between symptoms (Table II).

*Proteus* spp. bacteremia was caused by *P. vulgaris* in five (5.2%) patients and by *P. mirabilis* in other patients (94.8%). In *P. vulgaris* bacteremia cases, four strains were isolated only from blood samples. One *P. vulgaris* bacteremia was related with a previous isolation of the same species strain from a chronic wound.

Median age for all patients with *Proteus* spp. bacteremia was 71 years. We found that 14-day mortality ratio of *Proteus* spp. bacteremia was 28.9%. None of *P. vulgaris* strain was related with short-term mortality. We observed that elderly patients have a higher risk of mortality (Table I).

During the period analyzed, the most commonly used antibiotic for empiric therapy of BSIs was imipenem. Amongst 16 patients treated with imipenem, three (20.0%) of them died within 14 days. All *P. mirabilis* strains isolated from blood samples of these patients were susceptible to imipenem. Piperacillin with tazobactam was used in 15 cases of BSIs, and ceftriaxone – in 13 cases. The 14-days mortality rate for piperacillin with tazobactam was 26.7%, and for ceftriaxone – 44.0%.

Primary bacteremia was reported in 40 (40.8%) cases. The source of *P. mirabilis* secondary bacteremia was confirmed in 58 (59.2%) cases. In patients with secondary bacteremia, the main source of infection was the urinary tract (27; 46.6%), wounds of skin or soft tissues (14; 24.1%). In nine (9.2%) cases, *Proteus* spp.

Table I
Demographic and clinical parameters of patients with Proteus spp. BSIs (n = 83).

| Variable | | Death in 14 days (n = 24) | Survival (n = 59) | *p*-value* |
|---|---|---|---|---|
| Age (years), median (min-max) | | 77.5 (55–94) | 67.0 (12–88) | 0.0025** |
| Sex | Men, No. (%) | 13 (54.2) | 33 (55.9) | 0.9229 |
| | Women, No. (%) | 11 (45.8) | 26 (44.1) | |
| Length of stay in hospital (days), median (min-max) | | 19.5 (1–139) | 38 (5–406) | 0.0142** |
| Length of stay in hospital before *Proteus* spp. BSI (days), median (min-max) | | 9.5 (0–138) | 7.5 (0–380) | 1.0000** |
| Primary bacteremia, No. (%) | | 11 (45.8) | 23 (39.0) | 0.7420 |
| Secondary bacteremia, No. (%) | | 13 (54.2) | 36 (61.0) | |
| Adequate empiric antibiotic therapy, No. (%) | | 16 (66.7) | 43 (72.9) | 0.7648 |
| More than one strain in blood culture, No. (%) | | 9 (37.5) | 20 (34.0) | 0.9537 |
| ESBL *Proteus* spp., No. (%) | | 4 (16.7) | 11 (18.6) | 0.9185 |
| Predisposing factors | Urinal catheter, No. (%) | 18 (75.0) | 44 (67.8) | 0.8117 |
| | Central Venus Catheter, No. (%) | 15 (62.5) | 28 (47.5) | 0.3168 |
| | Diabetes, No. (%) | 7 (29.2) | 10 (16.9) | 0.3419 |
| | Hospitalization during past half year, No. (%) | 13 (54.2) | 25 (42.4) | 0.4625 |
| | Cancer, No. (%) | 3 (12.5) | 4 (6.8) | 0.6784 |
| | Surgery during past half year, No. (%) | 9 (37.5) | 33 (55.9) | 0.2003 |
| | Cardio-vascular diseases, No. (%) | 15 (62.5) | 23 (39.0) | 0.0879 |
| | Chronic wounds, No. (%) | 7 (29.2) | 13 (22.0) | 0.6849 |

* $Chi^2$ test, ** U Mann-Whitney test

Table II
The strains isolated from patients with *P. mirabilis* (n = 93) and *P. vulgaris* (n = 5) bacteremia.

| Year | *Protteus* spp. bacteremia | Secondary *Proteus* spp. bacteremia | | | | | | Isolation of *Proteus* spp. not related directly to bacteremia | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Source of secondary bacteremia (strains isolated at the time/up to three days before isolation from blood sample) | | | | | Before bacteremia (more than three days) | | | | | After bacteremia | | | | |
| | | | Urinary tract | Respiratory tract | Wounds | Blood catheter | Peritoneum | Respiratory tract | Wounds | Urinary tract | Blood catheter | Peritoneum | Respiratory tract | Wounds | Urinary tract | Blood catheter | Peritoneum |
| 2009 | 12 | 8 (66.7%) | 7 | 1 | 0 | 0 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| 2010 | 12 | 8 (66.7%) | 4 | 0 | 2 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 4 | 1 | 2 | 0 |
| 2011 | 7 | 4 (57.1%) | 1 | 0 | 2 | 0 | 1 | 0 | 2 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 |
| 2012 | 4 | 4 (100.0%) | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 2013 | 14 | 8 (57.1%) | 2 | 2 | 2 | 2 | 0 | 3 | 2 | 1 | 0 | 0 | 0 | 2 | 0 | 1 | 1 |
| 2014 | 14 | 9 (64.3%) | 5 | 0 | 3 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 2015 | 9 | 4 (44.4%) | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| 2016 | 15 | 8 (53.3%) | 5 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| 2017 | 11 | 5 (45.5%) | 1 | 2 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 2 | 2 | 1 | 1 | 0 |
| Total | 98 | 58 (59.2%) | 27 | 8 | 14 | 7 | 2 | 4 | 9 | 7 | 1 | 1 | 5 | 11 | 5 | 5 | 2 |

bacteremia occurred three days prior to isolation of the same bacterial species from wound swab, and in seven (7.1%) – from urine samples (Table II). One or more weeks after *P. mirabilis* bacteremia, strains of the same species were isolated from wound swabs (11; 11.2%), central venous catheter samples (5; 5.1%) and urine collected by a catheter (5; 5.1%).

All *Proteus* spp. strains isolated in 2016 and 2017 (n = 26) were susceptible to meropenem. Most strains were susceptible to piperacillin with tazobactam (95.9%), ceftazidime (87.8%) and amikacin (86.7%) (Table III). More than half of the strains examined (51.0%) were resistant to co-trimoxazole. Resistance to ciprofloxacin was reported in 30 (30.6%) strains. All 40 strains isolated from primary bacteremia cases were susceptible to piperacillin with tazobactam. No significant differences in the antimicrobial susceptibility of strains isolated from primary and secondary bacteremia were found (Table III).

ESBLs were found in 18 (18.3%) out of 98 *Proteus* spp. strains. Among all ESBL positive strains, 6 (33.3%) were isolated in 2016, and 5 (27.8%) in 2017. None of the *P. vulgaris* strains produced ESBLs.

We compared the antimicrobial drugs susceptibility of ESBLs-positive (ESBLs(+)) and ESBLs-negative (ESBLs(–)) strains using the $chi^2$ test (Fig. 2). Among the ESBLs(–) strains, all (n = 80) were susceptible to piperacillin with tazobactam. The ESBLs(+) strains were more resistant to all penicillins tested with beta-lactamases inhibitors and cephalosporins. Among the ESBLs(+) strains, more strains were non-susceptible to ciprofloxacin and co-trimoxazole than among ESBLs(–) bacteria (Fig. 2).

**Susceptibility patterns.** The susceptibility patterns of isolates from different specimens of the same patient were similar, except of the isolate B3 that was genetically different from B1 and B2 and was more resistant to antibiotics than other two isolates collected from the same patient. Isolate J2, collected from urine four days before the bacteremia, were resistant to trimethoprim while J1 were susceptible to this antibiotic (Table IV).

ESBLs were detected in five (19.2%) *P. mirabilis* isolates from two patients (D and G), treated in the Intensive Care Unit (G) and the Cardiology Department (D). Isolates D1, D2, G1, G2 and G3 had the same DNA profiles.

**Biofilm formation.** The A value was equal to 0.0654, based on the formazan absorbance of blank sample. The mean values (± SD) of absorbance recorded for biofilms formed by the references strains, were as follows: for *E. coli* ATCC 35218 – 0.1031 ± 0.01, and for *S. aureus* ATCC 6538 – 0.6931 ± 0.03. The results obtained for clinical *P. mirabilis* strains were shown in Table IV.

Weak biofilms were formed by 17 (65.4%) isolates, moderate biofilms by four (15.4%), and strong by five (19.2%) isolates. Most of the strains (8; 72.7%) isolated from blood samples formed weak biofilms. Two of three isolates collected from chronic wounds prior to bacteremia were able to form stronger biofilms than strains collected from blood samples. Two isolates

Table III
Antibacterial agents (Aas) susceptibility of *Proteus* spp. strains (n = 98) isolated from bacteremia.

| Aas | Primary bacteremia Aas susceptibility | | | | | | $p^*$ | Secondary bacteremia Aas susceptibility | | | | | | Total Aas susceptibility | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S | | I | | R | | | S | | I | | R | | S | | I | | R | |
| | n | % | n | % | n | % | | n | % | n | % | n | % | n | % | n | % | n | % |
| AMC | 9 | 22.5 | 24 | 60.0 | 7 | 17.5 | 0.4588 | 16 | 27.6 | 28 | 48.3 | 14 | 24.1 | 25 | 25.5 | 52 | 53.1 | 21 | 21.4 |
| TZP | 40 | 100.0 | 0 | 0.0 | 0 | 0.0 | 0.1316 | 54 | 93.1 | 0 | 0.0 | 4 | 6.9 | 94 | 95.9 | 0 | 0.0 | 4 | 4.1 |
| CMX | 28 | 70.0 | 0 | 0.0 | 12 | 30.0 | 0.6037 | 45 | 77.6 | 0 | 0.0 | 13 | 22.4 | 73 | 74.5 | 0 | 0.0 | 25 | 25.5 |
| CTX | 31 | 77.5 | 0 | 0.0 | 9 | 22.5 | 0.3393 | 46 | 79.3 | 0 | 0.0 | 12 | 20.7 | 77 | 78.6 | 0 | 0.0 | 21 | 21.4 |
| CAZ | 35 | 87.5 | 2 | 5.0 | 3 | 7.5 | 0.1697 | 51 | 87.9 | 0 | 0.0 | 7 | 12.1 | 86 | 87.8 | 2 | 2.0 | 10 | 10.2 |
| FEP | 32 | 80.0 | 2 | 5.0 | 6 | 15.0 | 0.4254 | 48 | 82.8 | 4 | 6.9 | 6 | 10.3 | 80 | 81.6 | 6 | 6.1 | 12 | 12.2 |
| IMP | 30 | 75.0 | 8 | 20.0 | 2 | 5.0 | 0.6062 | 49 | 84.5 | 8 | 13.8 | 1 | 1.7 | 79 | 80.6 | 16 | 16.3 | 3 | 3.1 |
| GEN | 29 | 72.5 | 1 | 2.5 | 10 | 25.0 | 0.3662 | 46 | 79.3 | 1 | 1.7 | 11 | 19.0 | 75 | 76.5 | 2 | 2.0 | 21 | 21.4 |
| AMK | 33 | 82.5 | 3 | 7.5 | 4 | 10.0 | 0.6311 | 52 | 89.7 | 4 | 6.9 | 2 | 3.4 | 85 | 86.7 | 7 | 7.1 | 6 | 6.1 |
| CIP | 24 | 60.0 | 1 | 2.5 | 15 | 37.5 | 0.4336 | 38 | 65.5 | 5 | 8.6 | 15 | 25.9 | 62 | 63.3 | 6 | 6.1 | 30 | 30.6 |
| SXT | 20 | 50.0 | 0 | 0.0 | 20 | 50.0 | 0.3165 | 28 | 48.3 | 0 | 0.0 | 30 | 51.7 | 48 | 49.0 | 0 | 0.0 | 50 | 51.0 |

* *chi*$^2$ test – differences in the susceptibility of *Proteus* spp. strains isolated from primary and secondary bacteremia
S – susceptible, I – intermediate, R – resistant, AMC – amoxicillin + clavulanic acid, TZP – piperacillin with tazobactam, CXM – cefuroxime, CTX – cefotaxime, CAZ – ceftazidime, FEP – cefepime, IMP – imipenem, GEN – gentamicin, AMK – amikacin, CIP – ciprofloxacin, SXT – co-trimoxazole

from urine samples of catheterized patients formed moderate and strong biofilms, while isolates cultured from blood sample – weak biofilms. The isolates from venal catheters formed weak biofilms, similarly to isolates from blood samples.

**Dienes phenomenon results.** We found demarcation lines between strains J1 and J2, B1 and B3, and B2 and B3 (Table IV). Those strains were not genetically identical. Strain J1, from urine collected by a catheter, formed a stronger biofilm than J2 cultured from the blood sample. The B3 strain, cultured from the bedsore swab sample, formed a strong biofilm, in contrast to B1 and B2 isolates that formed a weak biofilm.

**Genetic similarity of *P. mirabilis* isolates.** Using PFGE we found that isolates collected from different clinical specimens were identical in 10 of 11 patients (Table IV). In five patients (K, G, H, E, and J), the same strains were found in other localizations before the bacteremia was confirmed.

Strains J1 and J2, isolated from patient J, were genetically different and formed biofilms with various capacity, but they presented similar susceptibility patterns.

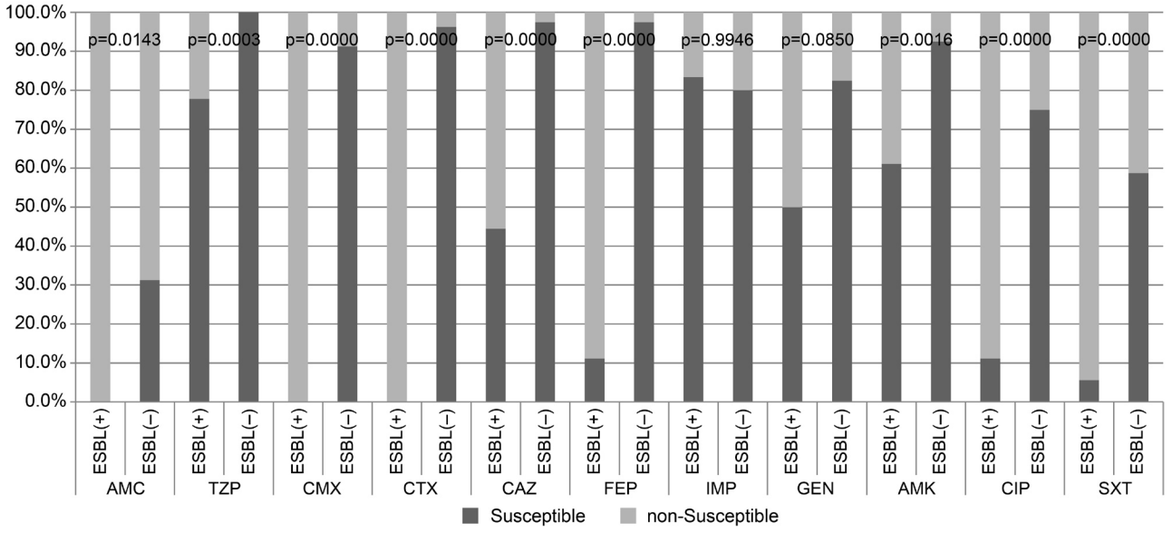
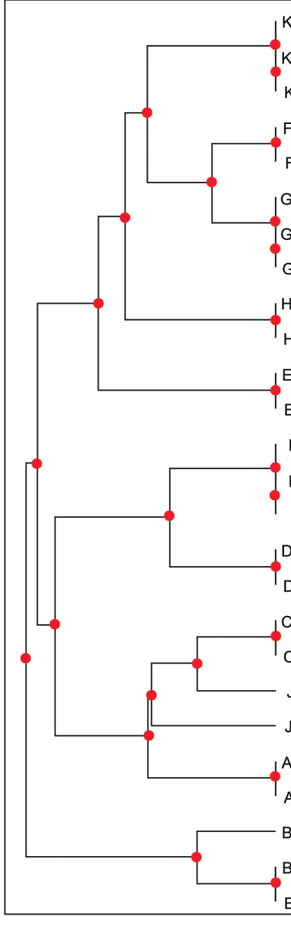



Fig. 2. Antibiotic susceptibility of ESBLs(+) and ESBLs(–) *Proteus* spp. strains (n = 98) isolated from bacteremia; *p*-value of *chi*$^2$ testing between susceptible and non-susceptible (resistant and intermediate) strains; abbreviations of antibiotic names (see Table III).

Table IV
Genetic similarity of *P. mirabilis* isolates (n = 26).




| | | | | AM | PIP | TIC | CEP | CXM | IMP | UNA | TIM | AMK | GEN | NET | TOB | CIP | NOR | TRM | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| K | S26/K3 | Bedsore swab | 11/02 | S | S | S | S | S | R | S | S | S | S | S | S | S | R | R | – | 0.185 ± 0.01 (M) | Compatible |
| | S25/K2 | Blood | 26/02 | S | S | S | S | S | I | S | S | S | S | S | S | S | I | R | – | 0.1137 ± 0.01 (W) | |
| | S24/K1 | Pus swab | 27/01 | S | S | S | S | S | I | S | S | S | S | S | S | S | I | R | – | 0.1147 ± 0.02 (W) | |
| F | S12/F2 | Blood | 24/09 | R | I | S | S | S | S | R | S | S | S | S | S | S | S | S | – | 0.1206 ± 0.04 (W) | Compatible |
| | S13/F1 | Urine by catheter | 15/10 | R | I | S | I | S | S | R | S | S | S | S | S | I | S | S | – | 0.2090 ± 0.02 (M) | |
| G | S14/G3 | Blood | 29/12 | R | R | S | R | S | S | R | S | S | S | S | S | S | I | R | + | 0.1156 ± 0.02 (W) | Compatible |
| | S15/G2 | Pus swab | 30/11 | R | R | S | I | S | S | R | I | S | S | S | S | I | I | R | + | 0.2511 ± 0.02 (S) | |
| | S16/G1 | Tissue | 30/1 | R | R | S | I | S | S | R | S | S | S | S | S | S | S | I | + | 0.1007 ± 0.04 (W) | |
| H | S18/H2 | Pus | 17/11 | I | S | S | S | S | S | S | S | S | S | S | S | S | S | R | – | 0.0989 ± 0.01 (W) | Compatible |
| | S17/H1 | Blood | 22/11 | S | S | S | S | S | S | S | S | S | S | S | S | S | S | I | – | 0.1112 ± 0.02 (W) | |
| E | S11/E2 | Bedsore swab | 30/06 | R | I | R | S | S | S | S | I | S | R | S | S | R | R | R | – | 0.8876 ± 0.05 (S) | Compatible |
| | S10/E1 | Blood | 06/07 | R | R | R | I | S | S | S | R | S | R | S | I | R | R | R | – | 0.7986 ± 0.04 (S) | |
| I | S19/I3 | Blood | 29/09 | R | R | R | S | R | S | R | S | R | S | S | S | S | S | R | – | 0.1212 ± 0.01 (W) | Compatible |
| | S20/I2 | Venous catheter | 28/09 | R | R | R | S | R | S | R | S | R | S | S | S | S | S | R | – | 0.1296 ± 0.02 (W) | |
| | S21/I1 | Pus | 15/10 | R | R | R | S | R | S | R | S | R | S | S | S | S | S | R | – | 0.1004 ± 0.02 (W) | |
| D | S9/D2 | Bedsore swab | 02/03 | R | R | R | I | R | S | R | R | S | R | S | I | R | R | R | + | 0.0956 ± 0.01 (W) | Compatible |
| | S8/D1 | Blood | 07/02 | R | R | R | R | R | S | R | R | S | R | S | R | R | R | R | + | 0.2197 ± 0.04 (M) | |
| C | S6/C2 | Blood | 30/04 | R | R | R | S | S | S | S | R | S | R | S | I | I | S | R | – | 0.0981 ± 0.01 (W) | Compatible |
| | S7/C1 | Venous catheter | 10/05 | R | R | R | S | S | S | S | R | S | R | S | R | I | S | R | – | 0.1126 ± 0.02 (W) | |
| J | S23/J2 | Urine by catheter | 14/11 | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | – | 0.4262 ± 0.02 (S) | Incompatible |
| | S22/J1 | Blood | 18/11 | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | – | 0.0887 ± 0.01 (W) | |
| A | S2/A2 | Urine | 13/03 | I | S | S | S | S | S | S | S | S | S | S | S | S | S | S | – | 0.1196 ± 0.03 (W) | Compatible |
| | S1/A1 | Blood | 14/03 | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | – | 0.1753 ± 0.04 (M) | |
| B | S5/B3 | Bedsore swab | 13/03 | R | R | R | S | S | S | I | R | S | R | S | R | S | S | R | – | 0.693 ± 0.02 (S) | Incompatible |
| | S4/B2 | Blood | 04/03 | R | S | S | S | S | S | I | R | S | R | S | S | S | S | R | – | 0.1032 ± 0.01 (W) | |
| | S3/B1 | BAL | 04/03 | R | S | S | S | S | S | I | R | S | R | S | S | S | S | R | – | 0.1125 ± 0.01 (W) | |

S – Susceptible, I – Intermediate, R – Resistant, abbreviations of antibiotics – in the text; *(S) – Strong, (M) – Moderate, (W) – Weak biofilms

The J2 strain was isolated from urine sample four days before J1 was cultured from blood sample.

Two strains, B1 and B2, were genetically identical as it was shown by their macrorestriction profiles. They formed weak biofilms and had the same susceptibility toward antibiotics. Strain B3, isolated from chronic wound swab nine days after confirmation of *P. mirabilis* bacteremia, was similar to B1 and B2, but it formed a strong biofilm and was resistant to higher number of the antibiotics tested than B1 and B2 isolates.

## Discussion

The *P. mirabilis* is isolated from 1% to 3% of all BSIs (Laupland et al. 2007; Sohn et al. 2011). Our study confirms these data. We also observed *Proteus* spp. BSIs in 2.5% of all BSIs investigated. Among *Enterobacterales*, *P. mirabilis* is the fourth Gram-negative bacteria species after *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter* spp. isolated from hospital-acquired BSIs (Sohn et al. 2011). BSIs are well-known cause of high mortality. We found that mortality rate of *P. mirabilis* BSIs was 28.9%. It is similar value to that obtained by Endimiani et al. (2005). They found that mortality rate attributable to *P. mirabilis* BSIs was 33.0%.

Laupland et al. (2014) showed that most cases of *P. mirabilis* bacteremia (18; 72.0%) have no documented sources and were recognized as primary bacteremia. In another seven (28.0%) cases, the urinary tract was confirmed to be the source of bacteremia. The opposite results were reported by Sohn et al. (2011). They detected primary bacteremia caused by *Proteus* spp. in 32.4% patients. The source of bacteria in secondary bacteremia was found mostly (35.1%) in the urinary tract and intra-abdominal cavity (21.6%) (Ahn et al. 2017). Infections of the urinary tract were admitted as the main source of BSIs also in the study by Tumbarello et al. (2012). They showed this phenomenon in BSIs caused by 19 (52.8%) multidrug resistant (MDR) and 28 (4.4%) non-MDR *P. mirabilis* strains.

Our data confirms the findings of Sohn et al. (2011). In most cases (58; 59.2%), the bacteremia caused by *P. mirabilis* was secondary due to the presence of these bacteria in other body sites, particularly in the urinary tract (27; 46.6%). In 14 (24.1%) patients, we found *P. mirabilis* in wound swabs. Wound infected by *P. mirabilis* was confirmed as the source of BSIs in the patient of the Traumatic Care Unit in India (Endimiani et al. 2005).

*Proteus* spp. is inherently susceptible to co-trimoxazole (trimethoprim-sulfamethoxazole). In the present study we found that more than half (51.0%) of the *Proteus* spp. strains, isolated from patients with bacteremia, were resistant to co-trimoxazole. High resistance to co-trimoxazole was reported also in another paper (Wang et al. 2014). Among 158 examined strains isolated from blood samples during longitudinal nationwide study from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) program, the resistance of *P. mirabilis* to co-trimoxazole was found in 65.2% strains. Similar percentage (69.2%) of co-trimoxazole resistance was reported also for ESBLs-producing *P. mirabilis* strains isolated from blood samples (Lebeaux et al. 2014). In this study, we found that almost all ESBLs(+) strains (17; 94.4%) were non-susceptible to co-trimoxazole. Lower percentages of resistant strains were found in the study by Tumbarello et al. (2012): 33.3% among non-MDR, and 30.6% among MDR *P. mirabilis* strains involved in BSIs development.

ESBLs were found by Sohn et al. (2010) in nine (24.3%) out of 37 *P. mirabilis* strains causing bacteremia from 2000 to 2009. In our study, we detected ESBLs in 18.4% *P. mirabilis* isolates causing bacteremia during the period evaluated. The highest number of ESBL-positive strains was found in 2016 (6; 33.3%). Tumbarello et al. (2012) noted that ESBLs were confirmed in 36 (36.4%) out of 99 *P. mirabilis* strains causing BSIs. MDRs were recorded for 36 (36.6%) strains tested.

Tumbarello et al. (2012) diagnosed *P. mirabilis* BSIs in 103 adult patients. The growth of the strains was inhibited by meropenem, amoxicillin with clavulanic acid, piperacillin with tazobactam, and amikacin. Ahn et al. (2017) confirmed that most *P. mirabilis* isolates from BSIs were susceptible to piperacillin with tazobactam (98.3%) and amikacin (93.5%). In the study by Wang et al. (2014), 58.9–87.3% of *P. mirabilis* strains isolated from blood samples were susceptible to gentamicin and amikacin, respectively. This is in agreement with the present results. *Proteus* spp. strains examined in this study were mostly susceptible to piperacillin with tazobactam (95.3%) and amikacin (86.7%).

*Proteus* spp. strains were generally susceptible to ciprofloxacin (67.1–73.4% of the strains) (Tumbarello et al. 2012; Wang et al. 2014). Tumbarello et al. (2012) found that susceptibility to ciprofloxacin is rare among MDR strains and was showed only by three (8.3%) out of 36 strains. Sohn et al. (2010) claim that a correlation between ciprofloxacin resistance and ESBLs production in *P. mirabilis* bacteremia may occur. This is in agreement with our results. We found significant differences in the susceptibility of ESBLs(+) and ESBLs(−) *Proteus* spp. strains to ciprofloxacin.

Sabbuba et al. (2003) investigated the genetic similarity of *P. mirabilis* strains isolated from hospitalized patients. They confirmed identical DNA profiles of the isolates from blood and urine of the same patient. In the present study, we report that strains isolated from blood samples of 10 out of 11 patients were identical with isolates cultured from other clinical specimens. The same strains were isolated from various specimens

also a few weeks after the bacteremia, that it is because of the survival of *Proteus* spp. strains despite the antibiotic treatment. The decrease in the effectiveness of antibiotic treatment may be related to the biofilm forming ability.

In most cases we observed that strains from clinical samples collected from infections related to biomaterials were able to form stronger biofilms than identical strains from other samples collected from these patients. The presence of biomaterials in body sites probably promotes biofilm formation, which is in agreement with the results obtained by Lebeaux et al. (2014) and Di Domenico et al. (2017). We also reported that isolates collected from chronic wounds prior to bacteremia were able to form stronger biofilms than isolates cultured from blood samples.

## Conclusions

In most cases bacteremia caused by *Proteus* spp. was secondary to urinary tract infection.

Most of the strains isolated from *Proteus* spp. BSIs were susceptible to piperacillin with tazobactam, third generation of cephalosporins, and amikacin.

Elderly patients have a higher risk of mortality in consequence of *Proteus* spp. BSI.

Most strains isolated from blood samples were identical to isolates cultured from other clinical specimens of the same patient.

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**Conflict of interest**

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

# Comparison of PCR, Fluorescent in Situ Hybridization and Blood Cultures for Detection of Bacteremia in Children and Adolescents During Antibiotic Therapy

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Abstract

The gold standard in microbiological diagnostics of bacteremia is a blood culture in automated systems. This method may take several days and has low sensitivity. New screening methods that could quickly reveal the presence of bacteria would be extremely useful. The objective of this study was to estimate the effectiveness of these methods with respect to blood cultures in the context of antibiotic therapy. Blood samples from 92 children with sepsis were analyzed. Blood cultures were carried out in standard automated systems. Subsequently, FISH (Fluorescent In-Situ Hybridization) and nested multiplex-real-time-PCR (PCR) were performed. Blood cultures, FISH and PCR yielded positive results in 18%, 39.1%, and 71.7% of samples, respectively. Significant differences were found between the results obtained through culture before and after induction of antibiotherapy: 25.5% vs. 9.7%. There was no significant difference in FISH and PCR results in relation to antibiotics. The three methods employed demonstrated significant differences in detecting bacteria effectively. Time to obtain test results for FISH and PCR averaged 4–5 hours. FISH and PCR allow to detect bacteria in blood without prior culture. These methods had high sensitivity for the detection of bacteremia regardless of antibiotherapy. They provide more timely results as compared to automated blood culture, and may be useful as rapid screening tests in sepsis.

Key words: antibiotic therapy, FISH, PCR, sepsis

## Introduction

Bacteremia is defined as a confirmed presence of one or more bacterial species in the blood (Albur et al. 2016). Sepsis is defined as a life-threatening organ dysfunction resulting from an impaired regulation of the response to an infection caused mainly by bacteremia. Criteria of organ dysfunction are based on Sepsis Related Organ Failure Assessment (SOFA) and quick Sepsis Related Organ Failure Assessment (qSOFA) scores. In the case of suspicion of a systemic infection, sepsis is diagnosed given ≥ 2 points in SOFA score or in the presence of ≥ 2 clinical signs in qSOFA score, including low blood pressure (SBP ≤ 100 mmHg), high respiratory rate (≥ 22 breaths/min) or altered mental status (Glasgow coma scale < 15). To give a diagnosis, systemic inflammatory response syndrome (SIRS) criteria still come as useful, including leucocytosis, and also CRP and procalcitonin level.

Current recommendations are unfortunately only related to adults (Singer et al. 2016). The pediatric criteria thus far, largely based on SIRS, met with a lot of criticism similarly to adult criteria, since sepsis in children differs when it comes to clinical signs and symptoms, lab results and management (Goldstein et al. 2005; Churpek et al. 2015; Kaukonen et al. 2015). Implementation of new sepsis recommendations in pediatrics needs standardization of organ failure depending on age, which is especially difficult owing to the specific character of childhood diseases.

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At present, it is considered that sepsis is the main cause of death in children globally (Dugani and Kissoon 2017). Epidemiologic data are incomplete, but it is estimated that infectious conditions are responsible for approximately 40% of deaths in children below 5 years of age. In 2015, the World Health Organization (WHO) has announced four main causes of death due to infectious conditions in children: pneumonia (16%), diarrhea (9%), neonatal sepsis (7%), malaria (5%), followed by measles (1%) and HIV/AIDS (1%) (WHO 2017).

Owing to limitations of the diagnostic criteria, it is still prevalent to identify the blood infection microbiologically, in order to definitely confirm sepsis.

The current "gold standard" of microbiological sepsis diagnostics is blood culture in automated systems. This method is of relatively small efficiency due to the low number of pathogens in the blood and the fact that it is time-consuming (up to 72 h), with 15–20% of results being positive (Jamal et al. 2006). Microbial growth inhibitors may additionally impede or delay the detection of microorganisms; therefore, it is recommended to administer adequate antibiotic therapy after blood for microbiological testing has been collected, but only if such collection will not significantly delay treatment (> 45 min). Lack of specific pathogen identification yields empirical therapy. Ineffectiveness of such treatment may be life threatening or leads to permanent multi-organ dysfunction and failure to thrive.

There are ongoing efforts to improve the efficacy of microbial identification with alternative diagnostic techniques, independent of antibiotic therapy, such as serological (detection of lipopolysaccharide (LPS) of Gram-negative bacteria or mannan and galactomannan of fungi) or molecular methods FISH and PCR, detecting the DNA or RNA of pathogens in the blood. Unfortunately, molecular methods are costly, need special laboratory apparatuses but are quick (3–4 h) and sensitive (Klouche and Schröder 2008; Gosiewski, Jurkiewicz-Badacz, et al. 2014; Źródłowski et al. 2017). Identification of the etiological agent and prompt targeted therapy could lower mortality and permanent health consequences in patients.

The aim of the study was the evaluation of efficacy of FISH, PCR and blood culture in detection of the etiological factor of bacteremia in children and adolescents, before and after antibiotic treatment.

## Experimental

### Materials and Methods

Blood samples from 92 children and adolescents aged from 1 week to 18 years of age (mean age 4.7 years; standard deviations (SD) 3.54), with the clinical symptoms and blood lab results of sepsis, were analysed. Patients in the age group 2 to 5 years dominated (45%), followed by neonates (23.4%).

Blood samples originated from the Department of Neuroinfections and Pediatric Neurology as well as from the Department of Pediatric Infectious Diseases of the John Paul II Specialist Hospital in Cracow in the years 2010–2012 (30 months) according to SIRS criteria. The study was approved by the Bioethical Committee from the Regional Chamber of Physicians in Cracow, decision no. 30/KBL/OIL/2010 from 17 March 2010.

Inclusion criteria (at least two were to be met) (Levy et al. 2003) were as follows: i) body temperature over 38.5°C or below 36.0°C (rectal or oral); ii) leukocytosis over 2 SD for the given age; iii) clinical signs and symptoms or results of additional studies suggesting an infection (organ abscess, leukocytes present in physiologically sterile fluids, radiological evidence of pneumonia, hemorrhagic eruption), and iv) C-reactive protein (CRP) exceeding 50 IU/ml.

The patients excluded from the study group consisted of those that did not fulfil the inclusion criteria, or no permission was obtained from their lawful caretakers. Also excluded were the patients whose blood samples were of too small volume to conduct at least one of the test methods. Eventually, 92 patients were included into the study.

**Microbiological examinations.** Two blood samples were taken from each patient. The samples came from a separate puncture of a different vein. Sample volume depended on the patient's age. In neonates: 1–2 ml of blood was collected, in children from 1 month to 2 years of age: 2–3 ml, in older children: 3–5 ml, in teenagers: 10 ml. Blood samples underwent standard microbiological diagnostics based on the BactAlert (bioMérieux, Marcy l'Etoile, France) automated blood culture system in the Microbiology Laboratory of the John Paul II Specialist Hospital in Cracow. The incubation time used for detection of bacteria in automated blood culture was 7 days.

**Molecular studies.** Parallel molecular studies were performed on the blood samples using FISH and PCR. In order to perform molecular studies, 1.5 ml of blood was collected to Vacutainer $K_3$EDTA tubes (BectonDickinson, Warsaw, Poland) from all patients, irrespective of age. Samples were frozen to –70°C, and then transferred to the Chair of Microbiology of Jagiellonian University Medical College in Cracow.

Microbial DNA isolation from blood. With the aim of determining the sensitivity of the PCR method, microbial DNA was isolated from 1.5-ml blood samples. DNA isolation was carried out according to the method described by Gosiewski et al. with the employment of a ready-to-use Blood Mini (A&A Biotechnology) kit (Gosiewski, Szała, et al. 2014).

PCR amplification. All the processes of DNA amplification were performed with the use of the real-time PCR method (qPCR) in a CFX96 thermal cycler (Bio-Rad) by employing the species-specific starters and TaqMan probes (Table IV), according to the procedure by Gosiewski et al. (Gosiewski, Flis, et al. 2014). Additionally, β-actin gene detection was performed in every sample of DNA isolated from blood in order to verify whether PCR inhibition takes place (Gosiewski, Jurkiewicz-Badacz, et al. 2014).

FISH method. 200 µl of blood was subjected to preparation using 0.17 M of ammonium chloride solution (ICN Biomedicals) as in the case of preparing blood samples for DNA isolation until a pale pink pellet was obtained. The pellet was suspended in 20 µl of sterile deionized water from which 10 µl was transferred onto the surface of SuperFrost®Plus (Menzel-Glaser) microscope slide for hybridization in order to get a smear of approximately 10 mm in diameter. The preparation was dried under laminar flow and subsequently poured over with 500 µl of 4% paraformaldehyde (Sigma) solution and incubated for 20 min at 4°C. Then, the preparation was washed with PBS and poured over with 2 ml of 96% methanol (POCh). The whole specimen was kept under cover for 15 min at 20°C. Upon completion of the fixation process, methanol was washed off with warm (37°C) PBS solution and placed on 20 µl of diluted solution of lysozyme (1 mg/ml) (Sigma) and lysostaphin (0.05 mg/ml) (Sigma). It was incubated for 5 min at 37°C and then washed twice with sterile deionized water. Hybridization was performed with the use of single-chain oligonucleotide probes (Table IV) labelled with fluorochromes at 5' ends, targeted at the 16S rRNA conservative fragment typical for the studied group of bacteria, according to the protocol published by Gosiewski et al. (Gosiewski, Flis, et al. 2014).

**Statistical methods.** Chi-squared test, Mann-Whitney *U* test and analysis of variance (ANOVA) were used to compare the effect of antibiotic therapy on the effectiveness of culture, PCR and FISH. Fisher's exact test, two-tailed, was used to compare the effectiveness of methods (Gretl software ver. 1.9.4.). Verification of statistical hypotheses was performed at a significance level of $p < 0.05$.

## Results

Data shown in Table I demonstrate that among the patients (n = 92) with blood samples collected and cultures performed before administration of antibiotics (n = 51), 25.5% of the samples were positive while 74.5% were negative. In the case of patients who had antibiotics administered prior to blood collection and blood cultures (n = 41), only 9.7% of the samples were positive and 90.3% negative – this difference was statistically significant. Gram-positive and Gram-negative bacteria constituted 47% (n = 8) and 53% (n = 9), respectively. In total, positive cultures were obtained from 18.5% (n = 17) of patients.

Table I
Antibiotic therapy vs. blood culture results.

| Blood culture | Antibiotic therapy prior to blood collection | |
|---|---|---|
| | n | % |
| Negative | 37 | 90.3 |
| Positive | 4 | 9.7 |
| Total | 41 | 100.0 |
| Blood culture | Antibiotic therapy after blood collection | |
| | n | % |
| Negative | 38 | 74.5 |
| Positive | 13 | 25.5 |
| Total | 51 | 100.0 |
| Blood culture | Total | |
| | n | % |
| Negative | 75 | 81.5 |
| Positive | 17 | 18.5 |
| Total | 92 | 100.0 |

$chi^2 = 5.768$; $p = 0.024$; Vc = 0.23; Results significant: $p < 0.05$

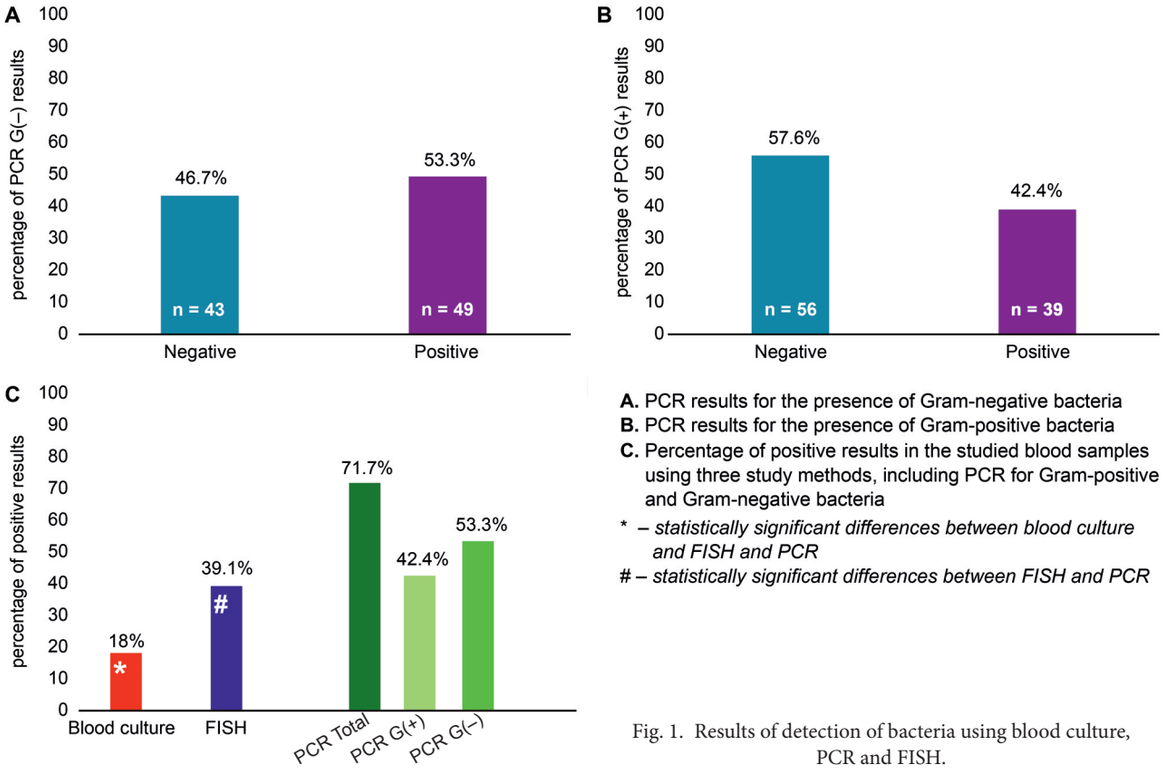
Table II
Antibiotic therapy vs. FISH results.

| FISH | Antibiotic therapy prior to blood collection | |
|---|---|---|
| | n | % |
| Negative | 31 | 63.3 |
| Positive | 18 | 36.7 |
| Total | 49 | 100.0 |
| FISH | Antibiotic therapy after blood collection | |
| | n | % |
| Negative | 25 | 58.1 |
| Positive | 18 | 41.9 |
| Total | 43 | 100.0 |
| FISH | Total | |
| | n | % |
| Negative | 56 | 60.9 |
| Positive | 36 | 39.1 |
| Total | 92 | 100.0 |

$chi^2 = 0.253$; not significant

FISH results were positive in 36 out of 92 (39.1%) patients and demonstrated the presence of Gram-negative rods (n = 4), which constituted 11.1%, Gram negative coccus (n = 4) – 11.1% and Gram-positive coccus (n = 28) – 77.8% of all the bacteria detected.

Table II shows the relationship between antibiotic therapy and the FISH results. Chi-squared test did not




**A.** PCR results for the presence of Gram-negative bacteria
**B.** PCR results for the presence of Gram-positive bacteria
**C.** Percentage of positive results in the studied blood samples using three study methods, including PCR for Gram-positive and Gram-negative bacteria

* – *statistically significant differences between blood culture and FISH and PCR*
\# – *statistically significant differences between FISH and PCR*

Fig. 1. Results of detection of bacteria using blood culture, PCR and FISH.

show any statistically significant differences between the attributes studied.

The PCR studies were performed for 92 patients in order to detect the presence of Gram-positive and Gram-negative bacteria. Amplification sensitivity was defined as the relation of the $C_T$ value, i.e. the number of reaction cycle in which the linear increase of the product cuts an established baseline 100 RFU (relative fluorescence unit) (Fig. 1). Table III shows the relationship between antibiotic therapy and PCR results. Chi-squared test did not show any statistically significant differences between the attributes studied.

Table III
Antibiotic therapy vs. PCR results.

| PCR | Antibiotic therapy prior to blood collection | |
|---|---|---|
| | n | % |
| Negative | 12 | 24.5 |
| Positive | 37 | 75.5 |
| Total | 49 | 100.0 |
| PCR | Antibiotic therapy after blood collection | |
| | n | % |
| Negative | 14 | 32.6 |
| Positive | 29 | 67.4 |
| Total | 43 | 100.0 |
| PCR | Total | |
| | n | % |
| Negative | 26 | 28.3 |
| Positive | 66 | 71.7 |
| Total | 92 | 100.0 |

$chi^2 = 0.731$; not significant

Figure 1 demonstrates positive results of the three methods used in this study: blood culture, FISH and PCR. Statistically significant differences were found between the results obtained after blood culture and FISH ($F = 0.0$, $P < 0.05$), FISH and PCR ($F = 0.0029$, $P < 0.05$) as well as blood culture and PCR ($F = 0.0108$, $P < 0.01$). Fungal microorganisms were not found with any of the methods. No significant differences in the effectiveness of the detection of the bacteria by three methods used depended on the age and sex of patients.

Repeatability of results using the three methods (culture, FISH and PCR) amounted to 21 samples (22.8%). Compatibility between blood culture and PCR involved 40 samples (43.5%), between blood culture and FISH: 71 samples (77.1%), while between FISH and PCR it was observed for 50 samples (54.3%).

## Discussion

The American College of Critical Care Medicine/Pediatric Advanced Life Support (ACCM/PALS) published sepsis management guidelines. The "sepsis bundles" were proposed to improve treatment efficacy. The

Table IV
Sequences of primers and probes (Genomed) utilized in this study.

| Amplification | Oligonucleotide | 5'-3' | Origin | Target sequences |
|---|---|---|---|---|
| Bacteria | EXT_BAC_F | kGCGrACGGGTGAGTAA | (Gosiewski, Jurkiewicz-Badacz, et al. 2014) | 16S rRNA |
| | EXT_BAC_R | CGCATTTCACCGCTA | | |
| | *GN/GP_F | GACTCCTACGGGAGGC | (Bispo et al. 2011) | |
| | *GN/GP_R | GCGGCTGCTGGCAC | | |
| | GP_Probe | Hex – CTGAyssAGCAACGCCGCG – TAMRA (Q) | | |
| | GN_Probe | Cy5 – CCTGAysCAGCmATGCCGCG – BHQ-2 | | |
| β-actin gene (amplification inhibition control) | F | GCCAGTGCCAGAAGAGCCAA | (Valle Jr et al. 2010) | Human β-actin gene |
| | R | TTAGGGTTGCCCATAACAGC | | |
| FISH | STA | CY3 – TCCTCCATATCTCTGCGC | (Kempf et al. 2000) | *Staphylococcus* spp. – 16S rRNA |
| | ENT 183 | CY3-5' – CTCTTTGGTCTTGCGACG | (Friedrich et al. 2003) | *Enterobacteriaceae* 16S rRNA |
| | EUB338 | FITC – GCTGCCTCCCGTAGGAGT – FITC | (Amann et al. 1990) | All bacteria – 16S rRNA |

main objectives were: hemodynamic stability, treatment of infection and lowering oxygen demand (de Oliveira et al. 2008). According to recommendations, intravenous antimicrobial therapy should be administered as early as possible, i.e. within 1 h of suspecting sepsis or septic shock. In a retrospective study, Kumar et al. (2006) have shown that early administration of appropriate antibiotics improved survival rates in adult patients with septic shock. Weiss et al. (2014) obtained similar results for children.

The success of therapy depends to a big extent on the identification of the etiological factor (Weinstein et al. 1983; Ibrahim et al. 2000; MacArthur et al. 2004). Presently, the "gold standard" for diagnosis of sepsis is a classic blood culture. Surely, the advantages of this method are its simplicity, low cost and the possibility to determine drug sensitivity of pathogens. The disadvantages of the method are the time consumption and low sensitivity. The average time from the blood collection to results is 3–5 days, and the proportion of positive cultures to the total number of cultures performed varies from 15 to 30%, depending on the studies (Jamal et al. 2006; Gosiewski, Flis, et al. 2014). Microbial growth inhibitors in the form of antibiotics play a major role in the negative culture results, what was shown in our study. Positive culture results were obtained from 25.5% of patients without prior antibiotic therapy, and from only 9.7% after antibiotic use (Table I). Owing to such results, the search for new techniques to identify pathogens in the blood is ongoing. FISH and PCR are relatively well known molecular techniques. FISH is generally used to study post-culture media samples (Farina et al. 2012). Presently, the method of sample purification was worked out by Gosiewski et al. and a much clearer high-quality microscopic view was obtained without auto-fluorescence in the background, which allows the blood examination without the need for prior cultures (Gosiewski, Flis, Sroka, Kędzierska, et al. 2014). The PCR method is used, among others, in the commercially available Septi-Fast test (Roche). The study is based on the PCR technique and allows to identify 25 most common microorganisms responsible for over 90% of sepsis cases. In our study, a nested-multiplex real-time PCR method worked out by Gosiewski et al. was used (Gosiewski, Jurkiewicz-Badacz, et al. 2014; Gosiewski, Flis, et al. 2014).

In our study, both molecular methods turned out to be more sensitive and quicker than the classic culture and independent of antibiotic therapy. FISH and PCR were performed in 92 patients and positive results were obtained in 39.1% and 71.7% of patients, respectively, whereas using the classical blood culture approach – only in 18.5% the bacteria were detected In some samples analysed, DNA of Gram-negative and Gram-positive bacteria was isolated simultaneously (42.4% versus 53.3%, respectively). Hence, the sum of results is higher than the positive results of PCR (71.7%). The difference in sensitivity of molecular tests vs. blood cultures results from the fact that cultures detect only living bacteria, which are able to grow, but molecular methods also confirm the presence of DNA that may originate from dead cells (Farina et al. 2012). Repeatability of results using the three methods was 22.8%. The calculation takes into account both consistent positive results as well as consistent negative results. The highest result repeatability (77.1%) was obtained when comparing blood cultures with FISH; therefore, it can be assumed that this analytical method could

be the best alternative to blood culture. On the other hand, it should be noted that FISH sensitivity is not high and amounts to circa $10^3$ CFU/ml. Hence, it is possible that it was the reason of the highest compatibility with culture (Gosiewski et al. 2011). Furthermore, high percentage of Gram-positive bacteria may be due to contamination of the sample with skin bacteria during collection or processing, but non-template control (NTC) gave negative results in PCR. Unfortunately, we did not obtained consent of the bioethical commission, especially since the study also involved newborns. Our previous research showed that bacterial DNA could be detected by amplification method in the blood of healthy adult people but its taxonomic composition is completely different from the one seen in septic patients (Gosiewski et al. 2017). Also, these results are comparable to our previous studies (Gosiewski et al. 2005; Źródłowski et al. 2017). In the literature, there are no reports on using FISH to detect bacteria directly in the blood, except for the publications of our team; therefore, we cannot compare our results with other studies.

Molecular diagnostics of sepsis is a very hard task. Unfortunately, the methods of molecular biology encounter limitations while conducting microbiological diagnostic of the blood. The difficulty lies in isolating DNA matrix of a proper quality and of the highest possible concentration. Different species of bacteria, which may be biological agents inducing sepsis (sometimes polietiological) are characterized by varied susceptibility to cell lysis and, consequently, the propensity to efficient DNA isolation. An additional problem is constituted by the fact that the blood contains heme, which is a very strong inhibitor of DNA polymerases used in PCR (Opel et al. 2010). It is still the case that blood culture remains the basic diagnostic method, although theoretically a technical potential exists to detect pathogens with the molecular methods.

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**Conflict of interest**

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ORIGINAL PAPER

# Drug Susceptibility of Non-tuberculous Strains of *Mycobacterium* Isolated from Birds from Poland

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Abstract

Mycobacterioses are a constant problem in backyard poultry, as well as pet birds. To date, no evidence of direct transmission of atypical bacilli between humans has been demonstrated, but it cannot be ruled out that sick animals can be a source of infection for people in their environment. The aim of the study was to identify mycobacteria isolated from birds with diagnosed mycobacteriosis and to determine the susceptibility of mycobacterial isolates from these animals to antituberculous drugs most commonly used in the treatment of mycobacterial infections in humans. For drug susceptibility tests, drugs such as isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, capreomycin, cycloserine and ethionamide were used. A high degree of drug resistance was demonstrated, particularly in *Mycobacterium avium*. Isolates of *Mycobacterium xenopi* showed a relatively good susceptibility to the drugs tested. The drug resistance of *Mycobacterium genavense* has not been determined, but this mycobacterium was identified in ten cases, which is the second most frequent occurrence in the cases studied.

Key words: avian mycobacteriosis, mycobacteriosis, *Mycobacterium avium*, *Mycobacterium xenopi*, *Mycobacterium genavense,* drug susceptibility tests

## Introduction

The genus *Mycobacterium* consists of more than 180 species with validly published names (Parte 2014). Most of them are saprophytic species widely distributed in the environment, some of which are pathogenic to humans and animals, including birds (Tan et al. 2016). Tuberculosis caused by the *Mycobacterium tuberculosis* complex has been found in birds that had contact with humans. Although in most of these cases the source of infection was human, domestic birds not only can be infection vectors, but also can show symptoms typical of the open form of tuberculosis, which results in active mycobacteria release to the environment, sometimes for long time periods (Washko et al. 1998; Montali et al. 2001; Steinmetz et al. 2006; Peters et al. 2007; Ledwoń et al. 2008; Lanteri et al. 2011). In birds, mycobacteriosis caused by non-tuberculous mycobacteria (NTM) is definitely a more common problem than tuberculosis. Clinical manifestations of mycobacteriosis in birds include emaciation, depression and diarrhea along with a marked atrophy of the breast muscle. Tubercular nodules can be seen in the liver, spleen, intestine and bone marrow. Granulomatous lesions without calcification are a prominent feature. The disease is rare in the well-organized poultry sector due to improved farm practices, but occurs in backyard poultry, zoos and aviaries (Portaels et al. 1996; Shitaye et al. 2008; Dhama et al. 2011; Pfeiffer et al. 2017). Mycobacterioses also pose a constantly and increasingly growing threat to human health, especially in people with weakened immunity (Schitaye et al. 2008; Slany et al. 2016; Titmarsh 2017). The mycobacteria most frequently isolated from birds with mycobacteriosis are *Mycobacterium avium* and *Mycobacterium genavense*. These bacteria are also a frequent cause of disease in people infected with Human Immunodeficiency Virus (HIV) (Ristola et al. 1999) or those receiving immunosuppressive treatment after transplantation or other systemic diseases (Tortoli 2006; Lastours de et al. 2008; Tan et al. 2016). A study

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conducted by Garcia-Marcos et al. (2017) showed a correlation between the occurrence of mycobacteriosis in children and their exposure to chickens. But NTM can also cause disease in adult immunocompetent patients (Piersimoni and Scarparo 2006; Santos et al. 2014). Because backyard poultry, pet birds and synanthropic animals live in the immediate vicinity of humans, it is important to study strains with potential pathogenicity for humans, as well as to assess the risk posed by their antibiotic resistance.

## Experimental

### Materials and Methods

The 46 samples tested (Table I) were collected over ten years from 37 dead and nine live birds originating mainly from Warsaw (Poland) and nearby areas. In the necropsied birds, except for one case, when the cause of death was a mechanical injury, advanced mycobacteriosis was diagnosed as the main cause of death. From the necropsied animals, liver samples were collected for microbiological and molecular examination. Samples from living birds were taken from areas affected by lesions associated with the disease. In three peafowl with symptoms of dyspnoea, tracheal swabs were collected. In two falcons, samples from abscesses localized within the subcutaneous tissue of the legs were collected. Faecal samples were taken from canaries and finches. These birds came from breeding sites in which cases of canary death due to mycobacteriosis had been reported earlier. Microscopic examinations of individually collected faeces from these aviaries allowed identification of carriers. Moreover, the presence of leukocytosis with neutrophilia and monocytosis confirmed active infection in those birds.

**Identification of mycobacteria.** All samples were stained with TB Ziehl-Neelsen KIT (QCA, Spain). Decontamination of clinical and dissected material was carried out using Sputofluol® (Merck, Germany), in accordance with the manufacturer's instruction. An initial culture of samples was carried out for up to 10 weeks at 37°C on the Löwenstein-Jansen PACT medium (Becton Dickinson, USA) and then up to six weeks on Bactec MGIT 960 (Becton Dickinson, USA).

Table I
Microorganisms present in the materials taken from birds with recognized mycobacteriosis.

| Avian species with diagnosed mycobacteriosis | No. of birds/AFB + samples | No. of cultured samples | Mycobacterium species (No. of isolates) |
|---|---|---|---|
| Ornamental chickens (*Gallus gallus*) | 8/8 | 8 | *Mycobacterium avium* (8) |
| Indian peafowl (*Pavo cristatus*) | 5/3 | 5 | *Mycobacterium avium* (5) |
| Pheasants (*Phasianus* spp.) | 5/5 | 4 + 1* | *Mycobacterium avium* (5) |
| Turkey (*Melleagris gallopavo*) | 1/1 | 1 | *Mycobacterium avium* (1) |
| Red-fronted parakeet (*Cyanoramphus novaezelandiae*) | 3/3 | 2<br>1 | *Mycobacterium avium* (2)<br>*Mycobacterium xenopi* (1)* |
| Barred parakeet (*Bolborhynchus lineola*) | 1/1 | 1* | *Mycobacterium genavense* (1) |
| Fischer's lovebird *(Agapornis fischeri*) | 1/1 | 1 | *Mycobacterium xenopi* (1) |
| Budgerigar (*Melopsittacus undulatus*) | 1/1 | 1* | *Mycobacterium genavense* (1) |
| Cockatiel (*Nymphicus hollandicus*) | 1/1 | 0 | *Mycobacterium genavense* (1) |
| Atlantic canary (*Serinus canaria*) | 5/5 | 2*<br>1** | *Mycobacterium genavense* (5)<br>*Mycobacterium xenopi* (1) |
| Goldfinch (*Carduelis carduelis*) | 1/1 | 0 | *Mycobacterim spp.* (1) |
| Bengalese finch (*Lonchura striata domestica*) | 2/2 | 1*<br>1 | *Mycobacterium genavense* (1)<br>*Mycobacterium* spp. (1) |
| Common buzzard (*Buteo buteo*) | 2/2 | 1<br>1* | *Mycobacterium avium* (1)<br>*Mycobacterium genavense* (1) |
| Peregrine falcon (*Falco peregrinus*) | 2/2 | 2 | *Mycobacterium avium* (2) |
| Rock pigeon (*Columba livia*) | 3/3*** | 3 | *Mycobacterium avium* (3) |
| Common wood pigeon (*Columba palumbus*) | 2/2 | 2* | *Mycobacterium avium* (2) |
| Rook (*Corvus frugilegus*) | 2/2 | 2 | *Mycobacterium avium* (2) |
| Total: 46 | 45/43 | 40 | (46)** |

* Lack of growth on media for drug resistance testing
** In one necropsied canary coinfection with *M. genavense* and *M. xenopi* were diagnosed
*** In one case of mycobacteriosis in a pigeon flock, *M. avium* was isolated from 19 individuals, but as all isolates were the same strain; therefore, they are considered as one case on the list

Positive cultures were investigated using the BD MGIT TBc Identification Test (TBc ID, Becton Dickinson, Sparks, MD), which is an immunochromatographic assay for a rapid discrimination between the *M. tuberculosis* complex and NTM. The TBc ID test is based on the detection of the antigen MPT64, one of the predominant proteins secreted by the *M. tuberculosis* complex strains during culture (Martin et al. 2011). The NTM samples were then tested using the GenoType Mycobacterium CM VER 2.0 test, a molecular genetic assay for the identification of clinically relevant mycobacterial species from mycobacterium contaminated material (Hain Lifescience GmbH, Germany).

Samples that did not grow on the Löwenstein-Jansen medium (Becton Dickinson, USA) were cultured onto the Middlebrook 7H9 (Becton Dickinson, USA) liquid medium supplemented with 2 mg/l Mycobactin J (Allied Monitor, Fayette, USA) (Coyle et al. 1992) for 20 weeks. Identification of samples from which no culture was obtained or were grown on the media with Mycobactin J was performed using SYBR® Green real-time PCR specific for *M. genavense*. DNA isolation from the liver tissue or bacterial cultures was performed using the GeneMATRIX Tissue & Bacterial DNA Purification Kit (Eurx®, Poland), while DNA isolation from faeces was made using the GeneMATRIX Stool DNA Purification Kit (Eurx®, Poland). The chosen forward primer MG 25-s(GAATCCGCTGCTGCTCTG) was located at nucleotides 378 to 395 of a *M. genavense* hypothetical 21 kDa protein gene (Chevrier, et al. 1999), while the backward primer MG25-as (TCAATGTAGTCCTGTCCGAAC) corresponded to nucleotides 313 to 291. Real-time PCR amplification was carried out in a total volume of 25 µl using SG qPCR Master Mix (Eurx®, Poland) according to the methodology described by the authors earlier (Ledwoń et al. 2009).

**Drug susceptibility testing**. Drug susceptibility tests were conducted using 34 mycobacterial isolates, including 31 *M. avium* subsp. *avium* and three *Mycobacterium xenopi*. Determination of susceptibility to drugs was performed by using the proportion method on the Löwenstein-Jensen and Middlebrook 7H9 media (Canetti et al. 1969; Zwolska et al. 1999; Augustynowicz-Kopeć et al. 2003). The susceptibility to isoniazid, rifampicin, streptomycin, ethambutol, ofloxacin, capreomycin, cycloserine and ethionamide was tested on the Löwenstein-Jansen medium. The susceptibility to clofazimine, rifabutin and sulfamethoxazole + trimethoprim was tested on the Middlebrook 7H9 medium. The concentrations of the antibiotics tested were as follows: streptomycin – 4.0, 8.0 µg/ml; isoniazid – 0.2, 0.4 µg/ml; rifampicin – 40.0, 80.0 µg/ml; ethambutol 2.0, 4.0 µg/ml; ofloxacin – 2.5, 5.0 µg/ml; capreomycin – 40.0, 80.0 µg/ml; cycloserine – 40.0, 80.0 µg/ml; ethionamide – 40.0, 80.0 µg/ml; clofazimine – 1.0, 2.0, 4.0 µg/ml; rifabutin – 0.5, 1.0, 2.0 µg/ml, and sulfamethoxazole + trimethoprim – 8.0, 16.0, and 32.0 µg/ml. Resistance was defined by the growth of ≥ 1% of a bacillary inoculum on the drug-containing medium compared to a drug-free control.

## Results

Mycobacteriosis was found in 46 birds belonging to 17 bird species (Table I). Gallinaceous birds were diagnosed with only *M. avium* infections, while pet birds were also diagnosed with *M. genavense* and *M. xenopi* infections. One dead canary was found to be infected with both *M. genavense* and *M. xenopi*. In wild birds, *M. avium* predominated, although *M. genavense* was found in one buzzard. Positive Ziehl-Neelsen staining (AFB +) was observed in 43 cases analysed. The growth of bacterial colonies on the Löwenstein-Jensen and Bactec MGIT 960 media was noticed for 34 samples collected. 31 isolates of *M. avium* and three isolates of *M. xenopi* were cultured. Drug susceptibility was tested for 28 *M. avium* and three *M. xenopi* strains (Table II and Table III). *M. avium* strains from common pheasant (*Phasianus colchicus*) and wood pigeons (*Columba palumbus*) did not demonstrate any growth on the media used for the assessment of drug resistance. Ten samples were identified as *M. genavense*, five of which were cultured on the Middlebrook 7H9 medium with

Table II
Drug resistance of 28 strains of *M. avium* isolated from birds.

| Drug* | SM | INH | RFP | EMB | CPM | OFLOX | CS | ETH | SXT | CFM | RIF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Susceptible | 8 | 0 | 0 | 0 | 20 | 0 | 25 | 3 | 8 | 27 | 10 |
| Moderately susceptible | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 0 |
| Moderately resistant | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 8 |
| Resistant | 4 | 28 | 28 | 28 | 5 | 28 | 2 | 0 | 4 | 1 | 10 |
| No data** | 16 | 0 | 0 | 0 | 3 | 0 | 1 | 25 | 0 | 0 | 0 |

* SM – streptomycin, RFP – rifampicin, INH – isoniazid, EMB – ethambutol, CPM – capreomycin, OFLOX – ofloxacin, CS – cycloserine, ETH – ethionamide, SXT – sulfamethoxazole + trimethoprim, CFM – clofazimine, RIF – rifabutin

** Number of strains which did not demonstrate any growth on the media used for the assessment of drug resistance

Table III
Drug susceptibility of three *M. xenopi* strains isolated from pet birds.

| Drug* | SM | INH | RFP | EMB | CPM | OFLOX | CS | ETH | SXT | CFM | RIF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Susceptible | 1 | 0 | 2 | 2 | 3 | 3 | 2 | 3 | 3 | 3 | 3 |
| Resistant | 0 | 3 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| No data** | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |

* SM – streptomycin, RFP – rifampicin, INH – isoniazid, EMB – ethambutol, CPM – capreomycin, OFLOX – ofloxacin, CS – cycloserine, ETH – ethionamide, SXT – sulfamethoxazole + trimethoprim, CFM – clofazimine, RIF – rifabutin
** Number of strains which did not demonstrate any growth on the media used for the assessment of drug susceptibility

Mycobactin J. Two samples were identified as *Mycobacterium* spp. These mycobacteria were also incapable of growing on the medium for susceptibility testing.

## Discussion

*M. avium* and *M. genavense* have been the main causes of mycobacterioses described to date in birds (Tell et al. 2001; Shivaprasad and Palmieri 2012; Pfeffer et al. 2017). Our study confirmed the results published by other authors. Mycobacterioses caused by *M. xenopi* are very rarely diagnosed in birds (St-Jean et al. 2018). In our investigations, even though only three isolates were obtained, it should not be underestimated. *M. xenopi* has recently been isolated from water (Slozarek et al. 1993) which is also a source of infection for humans and animals. Birds that were diagnosed with this bacterial infection, however, came from various breeders, so it could not be concluded that they infected themselves with the same water intake.

*M. genavense* is very difficult to be cultured on the media, and its diagnostics are mainly based on molecular methods (Hoop et al. 1993; Portaels et al. 1996; Ledwoń et al. 2009; Palmieri et al. 2013). Therefore, the drug susceptibility tests used in this study may not be useful for testing of these mycobacteria. Strains of *M. avium* (Table II) demonstrated 100% resistance to isoniazid, rifampicin and ethambutol, while strains tested on ethionamide showed a good susceptibility to that antibiotic. These results differ from those described in a study by Parvandar et al. (2016) in which all *M. avium* strains isolated from pigeons for drug susceptibility tests were shown to be resistant to streptomycin, kanamycin, ethionamide and thiophene carboxylic acid hydrazide. Moreover, single isolates were susceptible to isoniazid, rifampicin and ethambutol (Parvandar et al. 2016). In the cases described by Stepień-Pyśniak et al. (2016), *M. avium* strains isolated from birds demonstrated resistance to isoniazid, rifampicin, ethambutol, ethionamide, capreomycin and ofloxacin, and were susceptible to streptomycin and cycloserine. *M. xenopi* isolated from birds (Table III) presented resistance similar to that of isolates originating from humans (van Ingen et al. 2010). These isolates were resistant to isoniazid, slightly less resistant to rifampicin and ethambutol, and fully susceptible to other antibiotics. All three species of mycobacteria identified from the cases of mycobacteriosis in birds are able to cause mycobacteriosis in humans, particularly in immunocompromised hosts (Coyle et al. 1992; Ristola et al. 1999; Bluth et al. 2009; Shah et al. 2016; Adzic-Vukicevic et al. 2018). Therefore, mycobacterioses in birds living in a direct proximity to humans should be taken seriously, since they can constitute a potential reservoir of infection with mycobacteria resistant to multiple antimycobacterial drugs.

**Conflict of interest**

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ORIGINAL PAPER

# Evaluation of *Neisseria meningitidis* Carriage with the Analysis of Serogroups, Genogroups and Clonal Complexes among Polish Soldiers

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Abstract

*Neisseria meningitidis* is an etiological factor of invasive meningococcal disease (IMD). This Gram-negative diplococcus is transmitted from person to person via droplets or through a direct physical contact with secretions of infected patients or asymptomatic carriers. The latter account for 5–10% of the general population. The aim of the study was to estimate the actual *N. meningitidis* carriage rate in the military environment with identification of serogroups, genogroups, sequence types and clonal complexes of the isolates detected among Polish soldiers. The study was conducted during winter seasons of 2015 and 2016 and involved 883 professional soldiers from the Armoured Brigade in Świętoszów, Poland. The material for testing were nasopharyngeal swabs obtained from study participants. The samples were tested using standard microbiological methods (culture, incubation, microscopy, biochemical and automated identification). *N. meningitidis* isolates were subjected to slide agglutination test (identification of serogroups), the bacterial DNA was extracted and allowed to determine genogroups, clonal complexes and sequence types. 76 soldiers were found to be carriers of *N. meningitidis*, they accounted for 8.6% of the study group. The meningococcal isolates mostly belonged to serogroup B. Sequence types ST-11439, ST-136, ST-1136 and the clonal complex 41/44CC were found to be predominant. Clonal complexes responsible for IMD were detected in 15.8% of carriers and 1.4% of the whole study participants. Carriage rates of *N. meningitidis* among Polish soldiers were found to be similar to those reported in the general population.

Key words: *Neisseria meningitidis*, serogroups, genogroups, clonal complexes, soldiers

## Introduction

*Neisseria meningitidis* is an etiological factor of invasive meningococcal disease (IMD), which is an acute, infectious illness characterized by a severe clinical course, even if antibiotics are administered at an early stage and intensive care is provided (Rosenstein et al. 2001). This Gram-negative diplococcus colonizes the nasopharyngeal mucosa and is transmitted from person to person via droplets or through a direct physical contact with secretions of infected patients or asymptomatic carriers, who account for 5–10% of the general population (Soriano-Gabarro et al. 2011). It is estimated, that carriage rates of *N. meningitidis* in closed environments (dormitories, military units) may be significantly higher and reach >40% (Tyski et al. 2000). Until now, twelve meningococcal serogroups have been described (they are distinguished according to biochemical constitution of the polysaccharide capsules of bacteria). The groups of *N. meningitidis* mostly associated with IMD are A, B, C, W and Y (Skoczyńska and Hryniewicz 2012). Serogroup incidence varies depending on the geographical area (Jafri et al. 2013). Groups B and C are a main cause of IMD in Americas and Europe. It is estimated that there are 1.2 million new IMD cases worldwide every year, with mortality reaching 10% or even 70% in cases of a septic shock (Rosenstein et al. 2001). In Poland, IMD incidence was found to be 0.43/100 000 in 2016 and 0.58/100 000 in 2017 (NIZP-PZH 2018). IMD cases have also been reported among the Polish military personnel: four cases in the military base in Skwierzyna, including two deaths in 2006 (Grecki and Bienias 2006), 15 cases in a military base in Warsaw, including two deaths in 2007 (Kadłubowski et al. 2007), and one death from IMD in the Polish Military Contingent in Afghanistan (Konior and Korzeniewski 2016). The incidence of IMD in US military personnel, historically far above that in the general population, has decreased >90% since in early 1970s, when the first vaccine against meningococcal infections was introduced (Broderick et al. 2015).

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Over the last decade, incidences of IMD in the military and US general population have become equivalent (Broderick et al. 2012).

Meningococcal carriage studies conducted among European conscripts over the last several decades demonstrated high carriage incidence, often exceeding 30%. The studies by Tyski et al. (2001) carried out in the last decade of the 20th century revealed that *N. meningitidis* carriage incidence among Polish conscripts was 60%. However, in 2009 Poland suspended conscription and transformed its national army into a fully professional organization. Recruits have been replaced by professional soldiers, whose socio-demographic profile as well as the character of work/service they perform is different from that of young conscripts who used to serve on a 24/7 basis and were permanently assigned to a given military facility. The first study presenting the general prevalence of *N. meningitidis* carriage in professional soldiers in Poland was conducted in winter season of 2016. The overall carrier rate among 1246 soldiers from the Armoured Brigade amounted to 5.2% (single examination), with the serogroup B being predominant (Korzeniewski et al. 2017).

The aim of the present study was to estimate the actual *N. meningitidis* carriage rate in the military environment with identification of serogroups, genogroups, sequence types and clonal complexes of the isolates (including clonal complexes responsible for IMD) detected among Polish soldiers.

## Experimental

### Materials and Methods

**Study population.** A total of 1766 biological samples from nasopharyngeal mucosa were taken from 883 professional soldiers serving in the 10th Armoured Brigade in Świętoszów, Poland (the first sample was collected in 2015, the second in 2016 from the same individual). The soldiers were joining the study group during the winter season (January–March); all of the participants had to provide informed consent and complete a socio-demographic and behavioral questionnaire including information on their military rank, age, sex, place of residence, cigarette smoking, medications taken on a regular basis, and vaccinations received against meningococcal serogroups A, C, W-135, Y (18% of examined soldiers were vaccinated between 2008 and 2014). Only professional soldiers, men or women aged 21–59 years (median 31 years), with 1–26 years of military service, in a good health condition were involved in the study. Soldiers with any anatomical or pathological lesions in the nasopharynx, which could prevent the collection of samples were excluded from the study.

**Ethical Procedure.** A research task was approved by the Ethics Committee of the Military Institute of Medicine in Warsaw, Poland (Decision No. 24/WIM/2014 of 18 Aug 2014).

**Identification of isolates.** The specimens obtained from the nasopharynx were transported to the microbiological laboratory in the Military Institute of Medicine, Warsaw, where they were plated onto the appropriate medium, i.e. Columbia Agar with 5% sheep blood and PoliVitex VCA3 and incubated under elevated $CO_2$ concentration at 37°C for 48 hours. After incubation, the colonies grown were macroscopically evaluated. Colonies morphologically similar to *N. meningitidis* phenotypes were isolated onto Columbia Agar with 5% sheep blood and were incubated under elevated $CO_2$ concentration at 37°C for 24 hours. The incubated pure colonies of bacteria were used to prepare Gram-stained preparations, which were then observed under a light microscope. Catalase and cytochrome oxidase tests were performed. All Gram-negative strains were identified based on their biochemical features using Vitek 2 NH card. The strains identified as *N. meningitidis* were stored frozen in a temperature of –20°C and then transported to the National Reference Center for the Diagnostics of Bacterial Infections of the Central Nervous System (KOROUN) in Warsaw, Poland for further diagnostics.

**Serogrouping.** The strains delivered to KOROUN were revived by placing them onto Columbia Agar medium; they were incubated in elevated $CO_2$ atmosphere at 37°C for 24 hours. Serogroups of all isolates were identified with a slide agglutination test using a set of primers in compliance with the manufacturer's instructions (detection of capsular antigens of *N. meningitidis*). The specific reagents covered the following serogroups: A, B, C, Y and W (Remel), E29 (Bio-Rad), X and Z (Becton Dickinson). Typing was performed on colonies isolated from the Mueller-Hinton agar medium following the identification of the strains.

**Genogrouping.** Chromosomal DNA was isolated from meningococcal isolates with Genomic DNA Prep Plus (A&A Biotechnology) following the manufacturer's recommendations. Genogroups were identified with the PCR assay using genogroup-specific oligonucleotide primers *orf-2*(A), *siaD*(C), *siaD*(W135) and *siaD*(Y) described by Taha (2000) and *siaD*(B) described by Guiver et al. (2000). The products were analyzed on the agarose gel. Genogroups A, B, C, E29, W, Y were identified.

**Sequencing.** Sequencing was performed on seven DNA fragments of the isolated strains. The chromatograms were analyzed using the Seqed 10.3 programme (Applied Biosystems) and the sequences obtained from the specimens were compared with the sequences available in the international database (http://pubmlst.org/

neisseria/). Individual loci were assigned to the right alleles. We identified the sequence type (ST) and clonal complex (CC) of the isolates which had a complete allelic profile (seven loci). In the case of isolates whose allelic profile was incomplete, only the clonal complex was identified.

**Statistics.** Statistical analysis was performed using STATISTICA PL version 12.0 and Microsoft Excel 2013. The quantitative variables were counted by arithmetic mean ± SD or median and 95% confidence interval. The qualitative variables were introduced in the absolute or percentage terms. Significance of differences between two groups was processed with the t-Student or U Mann-Whitney test. In all the calculations *p*-value under 0.05 was considered statistically significant.

## Results

A nasopharyngeal culture sample was collected twice during winter seasons (in 2015 and then in 2016) from 883 soldiers. 76 of the subjects tested were found to be carriers (at least one of the single samples collected in 2015 and 2016 was positive for *N. meningitidis*); the carriers accounted for 8.6% of the study group. In 2015, genogroups were determined for 46 isolates: B (n = 33, 61.1%), E29 (n = 6, 11.1%), C (n = 4, 7.4%), Y (n = 2, 3.7%), W (n = 1, 1.9%). Eight isolates were non-groupable (NG, 14.8%) with the primers used. In 2016, genogroups were determined for 46 isolates: B (n = 24, 45.3%), E29 (n = 8, 15.1%), C (n = 6, 11.3%), Y (n = 6, 11.3%), W (n = 2, 3.8%); genogroups of 7 isolates (NG, 13.2%) could not be determined with the primers used (Fig. 1).

Based on the MLST analysis, 21 different sequence types were identified among the isolates analyzed. Although the analysis was repeated several times, the sequence type and clonal complex of one of the isolates of genogroup B could not be determined in 2016. In both studies, 12 clonal complexes (CC) were identified among 62 *N. meningitidis* strains (25 isolates were identical to each other in both years, 12 were isolated once only). The most common clonal complexes included: 41/44CC (n = 6), 1136CC (n = 2), 53CC (n = 2). Only five strains which were found in both studies (in 2015 and again in 2016) and two strains isolated in 2016 were not assigned to any clonal complex. In six carriers the authors observed a change in the sequence type at the second collection and in four carriers also a change in the clonal complex. In one carrier genogroup B was found at the first collection and was replaced by genogroup Y at the second collection (Table I).

The mean age of *N. meningitidis* carriers in the study group was 30.4 ± 4.7 years and of non-carriers it was 32.1 ± 5.3 years. Carriers were significantly younger ($p = 0.0083$). No statistically significant differences were found between carriage prevalence and sex or residence. There were significantly more tobacco smokers among *N. meningitidis* carriers than in the non-carrier group (51.3% vs. 32.8%; $p = 0.0012$). The distribution of military ranks in both groups (carriers vs. non-carriers) was found to be statistically significant ($p = 0.0088$), the corps of privates being the largest. There were no statistically significant differences between carrier state and prior vaccination against *N. meningitidis* (Table II).

## Discussion

*N. meningitidis*, the etiological factor of invasive meningococcal disease (IMD), can be the cause of meningitis and/or sepsis. In most cases, meningococcal carriage does not lead to invasive disease, but is limited to asymptomatic carrier state which is normally found in 5–10% of the general population (Tzeng and

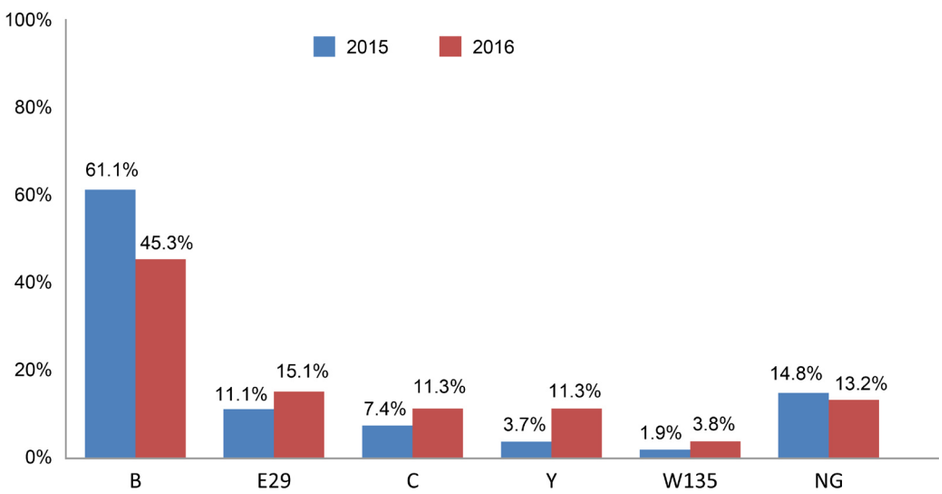


Fig. 1. Percentage distribution of *N. meningitidis* genogroups among Polish soldiers tested in years 2015–2016.

Table I
Distribution of sequence types (ST), clonal complexes and genogroups of the *N. meningitidis* isolates tested in years 2015–2016.

| ST 2015 | Clonal complex 2015 | ST 2016 | Clonal complex 2016 |
|---|---|---|---|
| Genogrup B | | | |
| 136 | ST-41/44 complex/Lineage 3 | 136 | ST-41/44 complex/Lineage 3 |
| 136 | | 136 | |
| 136 | | 136 | |
| 1097 | | 1097 | |
| 2840 | | 2840 | |
| 11442 | | 8107 | ST-213 complex |
| 1732 | | negative | negative |
| 112 | | negative | negative |
| 973 | | negative | negative |
| 35 | ST-35 complex | 35 | ST-35 complex |
| 35 | | negative | negative |
| 35 | | negative | negative |
| 35 | | negative | negative |
| 162 | ST-162 complex | 162 | ST-162 complex |
| 162 | | 4509 | – |
| 162 | | negative | negative |
| 162 | | negative | negative |
| 11433 | ST-213 complex | 11433 | ST-213 complex |
| 213 | | negative | negative |
| 33 | ST-32 complex | negative | negative |
| 33 | | negative | negative |
| 32 | | negative | negative |
| 11440 | ST-1136 complex | 11440 | ST-1136 complex |
| 2126 | ST-53 complex | 2126 | ST-53 complex |
| 198 | ST-198 complex | 198 | ST-198 complex |
| 1001 | ST-18 complex | 11446 | – |
| 11436 | ST-364 complex | negative | negative |
| 5133 | ST-103 complex | negative | negative |
| 9316 | | 9316 | – |
| 1572 | | 1572 | – |
| 11444 | | negative | negative |
| 11447 | | negative | negative |
| negative | negative | 35 | ST-35 complex |
| negative | negative | 11436 | ST-364 complex |
| negative | negative | 36 | – |
| negative | negative | 12187 | – |
| negative | negative | 9157 | – |
| negative | negative | 12186 | – |
| negative | negative | 120 | – |
| negative | negative | undetermined[1] | undetermined[1] |
| Genogrup E29 | | | |
| 11434 | ST-254 complex | 11434 | ST-254 complex |
| 11438 | | 11438 | – |
| 1138 | ST-60 complex | 1138 | ST-60 complex |
| 11439 | | 11439 | – |
| 11439 | | 11439 | – |

Table I continued

| ST 2015 | Clonal complex 2015 | ST 2016 | Clonal complex 2016 |
|---|---|---|---|
| 11445 | ST-60 complex | negative | negative |
| negative | negative | 1157 | ST-1157 complex |
| negative | negative | 60 | ST-60 complex |
| negative | negative | 11438 | ST-11438 complex |
| Genogrup C | | | |
| 2433 | ST-41/44 complex/Lineage 3 | 2433 | ST-41/44 complex/Lineage 3 |
| 2003 | – | 2003 | – |
| 5238 | – | negative | negative |
| 5133 | ST-103 complex | negative | negative |
| negative | negative | 3346 | ST-41/44 complex/Lineage 3 |
| negative | negative | 3346 | |
| negative | negative | 2433 | |
| negative | negative | 8108 | ST-174 complex |
| Genogrup Y | | | |
| 767 | ST-167 complex | 767 | ST-167 complex |
| 9316 | – | 9316 | – |
| negative | negative | 767 | ST-167 complex |
| negative | negative | 767 | |
| negative | negative | 3342 | ST-865 compex |
| Genogrup W | | | |
| 112 | ST-41/44 complex/Lineage 3 | 112 | ST-41/44 complex/Lineage 3 |
| negative | negative | 22 | ST-22 complex |
| NG | | | |
| 9268 | ST-53 complex | 9268 | ST-53 complex |
| 10159 | | 53 | |
| 53 | | negative | negative |
| 1136 | ST-1136 complex | 1136 | ST-1136 complex |
| 1136 | | negative | negative |
| 198 | ST-198 complex | negative | negative |
| 198 | | negative | negative |
| 11441 | ST-364 complex | 11441 | ST-364 complex |
| negative | negative | 4431 | ST-41/44 complex/Lineage 3 |
| negative | negative | 3461 | |
| negative | negative | 9466 | |
| Genogrup B / Genogrup Y[2] | | | |
| 11437 | ST-41/44 complex/Lineage 3 | 92 | ST-92 complex |

[1] Sequence type and clonal complex could not be determined
[2] Genogroup B was identified in the first collection and genogroup Y was detected in the second collection

Stephens 2000). In Poland, there have been reports of IMD cases in recent years; however, the actual carriage incidence of *N. meningitidis* among Polish residents has not been determined (Jafri et al. 2013). Many of Polish and European publications on the *N. meningitidis* carriage incidence are limited to military environment (Chapalain et al. 1992; Tyski et al. 2000; Korzeniewski et al. 2015). According to the research findings, meningococcal carriage is high among conscripted soldiers serving in European armies, whereas among professional soldiers it was found to be like the carriage rates observed in the general population (Korzeniewski et al. 2017). Meningococcal carriage studies conducted among military personnel also revealed that serogroup B was the most prevalent (Taha 2000). In Denmark, 43% of newly drafted recruits were found to be carriers of *N. meningitidis*, of whom 34% were transient carriers (this was associated with the colonization variability in the nasopharyngeal mucosa); 34% of the strains isolated from carriers belonged to serogroup B (Andersen et al.

Table II
Socio-demografic variables between control group (non-carriers) and carriers of *N. meningitidis* in years 2015–2016.

| Socio-demographic variables | Control group (non-carriers n = 807) | Carriers (n = 76) | p-value |
|---|---|---|---|
| **Age** | | | 0.0083 |
| Mean (SD) | 32.1 (5.3) | 30.4 (4.7) | |
| Range | 21.0–59.0 | 23.0–45.0 | |
| Median | 31.0 | 30.0 | |
| **Sex** | | | 0.6490 |
| Women | 65 (8.1%) | 5 (6.6%) | |
| Men | 742 (91.9%) | 71 (93.4%) | |
| **Place of residence** | | | 0.3318 |
| Rural area | 322 (39.9%) | 26 (34.2%) | |
| Urban area | 485 (60.1%) | 50 (65.8%) | |
| **Smoking cigarettes** | | | 0.0012 |
| Yes | 265 (32.8%) | 39 (51.3%) | |
| No | 542 (67.2%) | 37 (48.7%) | |
| **Respiratory tract infection** | | | 0.1106 |
| Yes | 61 (7.6%) | 2 (2.6%) | |
| No | 746 (92.4%) | 74 (97.4%) | |
| **Military rank** | | | 0.0088 |
| Private | 479 (59.4%) | 57 (75.0%) | |
| Non-commissioned officer | 276 (34.2%) | 19 (25.0%) | |
| Officer | 52 (6.4%) | 0 (0.0%) | |
| **Vaccination** | | | 0.1567 |
| Yes | 148 (18.3%) | 9 (11.8%) | |
| No | 659 (81.7%) | 67 (88.2%) | |

1998). The study, which was conducted in six military camps in Bavaria, found that 32% of the soldiers tested were carriers of *N. meningitidis*, and serogroup B was identified in 42% of them (Claus et al. 2005). Exceptionally high rates of meningococcal carriage, over 70%, were observed among British and Norwegian recruits, in whom serogroup B was also the most commonly found (Fraser et al. 1973; Caugant et al. 1992). There have been very few meningococcal carriage surveillance studies outside Europe. One of such studies, with the participation of Iranian soldiers, demonstrated that the incidence of *N. meningitidis* carriage among newly drafted soldiers was 11%, but after 2 months of service it increased to 33%. The study also revealed that serogroup B was predominant among the recruits tested (Eslami-Nejad et al. 2005). A study involving Iranian recruits which was performed a decade later demonstrated a lower carriage rate (8%), with a dominance of serogroup C (Ataee et al. 2016). A research study into Polish recruits carried out between 1998 and 1999 demonstrated meningococcal carriage rate at 31%, with the predominance of serogroup B (Tyski et al. 1998; Tyski et al. 2000). The first meningococcal carriage study in Polish professional soldiers (n = 559), which was conducted in 2013, showed the incidence of *N. meningitidis* carriage at 5.7% (Korzeniewski et al. 2015). A significant factor contributing to a reduction in meningococcal carriage incidence in the military environment was a change in the character of military service after the Polish Armed Forces have been transformed into a professional organization. Recruits used to serve on a 24/7 basis, they were permanently accommodated in the barracks and had all their meals in military dining facilities. Professional soldiers, on the other hand, typically work 8 hours a day and they are accommodated outside the military facilities (professional military service has changed to a regular job, with risk factors similar to those observed in the civilian environment). Another factor contributing to decreasing of meningococcal carriage prevalence was raise of the age of soldiers (19–20 year old recruits vs. professional privates who usually begin their military career at the age of 25–30 years old) (Korzeniewski et al. 2017). Meningococcal vaccination may also have a positive impact on reducing carriage prevalence of *N. meningitidis* and lowering the number of new IMD

cases; however, its effectiveness varies depending on the type of vaccine used, with conjugate vaccines being the most effective (Decker 2016). It needs to be pointed out that meningococcal vaccination does not completely prevent individuals from acquiring an infection, e.g. cases of the disease were reported among French soldiers, despite the use of immunoprophylaxis (Duron et al. 2016). The studies with the participation of Polish military personnel demonstrated that meningococcal carriage incidence was lower in older soldiers; the results are consistent with the available research findings (Taha 2000; Tzeng and Stephens 2000; Caugant et al. 2009; Soriano-Gabarro et al. 2011). Another factor associated with an increased risk for meningococcal carriage was found to be tobacco smoking (Blackwell et al. 1990; Caugant et al. 1992; Korzeniewski et al. 2017). The studies by Caugant et al. (1988) demonstrated that hypervirulent *N. meningitidis* strains rarely colonize the nasopharyngeal mucosa of carriers. The present study, and especially the application of sequencing methods, has proved to be useful in determining the genetic relationship among different strains and estimating the risk for IMD among carriers. The study involving Polish soldiers found virulent strains in 15.8% of carriers (n = 76) and 1.4% of the whole study group (n = 883). Stephens (1999) estimated that the risk of developing IMD by people living in closed environments is 1–1.5%. The incidence of specific clonal complexes is associated with the dominance of individual serogroups (Trotter et al. 2007; Jandova et al. 2016). In Poland serogroup B, comprising ST-32CC, ST-18CC, ST-41/44CC, ST-213CC and ST-269, was found to be predominant. Serogroup C covers ST-103CC, ST-41/44CC and ST-11CC. Serogroup W is represented by ST-22CC, and Y by ST-22CC, ST-23CC, ST-92CC, ST-167CC (Skoczyńska et al. 2013; Waśko et al. 2015). The present study confirmed that serogroup B was predominant among carriers; it was mostly represented by ST-41/44CC. Similar sequencing results were observed in Greece in a population of military recruits (Tryfinopoulou et al. 2016). Hypervirulent clonal complexes ST-213CC, ST-213CC, which have been observed in Poland, were also found in the study participants. Among serogroup C isolates the most common were clonal complexes ST-41/44CC and ST-103CC. Serogroup C was the third most common, after serogroup B and E29. Serogroup E29 was represented by the clonal complex ST-254CC and ST-60CC. At present, there is no data available concerning IMD morbidity from this particular serogroup (PubMLST, 2018). Serogroup Y covered the virulent clonal complex ST-167CC and once – ST-92CC. In this study, among carriers of serogroup W the clonal complex ST-22CC was identified, which was listed by the National Reference Center for the Diagnostics of Bacterial Infections of Central Nervous System in Warsaw as the strain responsible for causing IMD cases in Poland (Skoczyńska et al. 2013). In this current study, the hyperepidemic and hypervirulent strain ST-11 has not been identified. This strain is rarely found in carriers, its carriage is usually transient as the strain is rapidly transmitted to other hosts (Moxon and Jansen 2005). In general, there are significant differences in the incidence of the clonal complexes causing IMD and those responsible for meningococcal carriage (Caugant et al. 2007). Studies aimed at detecting pathogenic strains have been found to be valuable in determination of transmission routes, the distribution of individual genogroups and the epidemiological variability of *N. meningitidis*. While the surveillance of circulating strains is useful in selecting the best immunoprophylaxis (Millar et al. 2016).

## Conclusions

The overall carriage rates and types of serogroups of *N. meningitidis* among Polish professional soldiers were similar to the carriage reported in the general population. Clonal complexes responsible for IMD were detected in 15.8% of carriers and 1.4% of the whole study participants. Meningococcal carriage in professional soldiers was largely associated with a younger age, low military rank and frequent tobacco smoking.

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**Conflict of interest**

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## Literature

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ORIGINAL PAPER

# Epilithic Biofilms in Lake Baikal: Screening and Diversity of PKS and NRPS Genes in the Genomes of Heterotrophic Bacteria

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Abstract

A collection of heterotrophic bacteria consisting of 167 strains was obtained from microbial communities of biofilms formed on solid substrates in the littoral zone of Lake Baikal. Based on the analysis of 16S rRNA gene fragments, the isolates were classified to four phyla: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. To assess their biotechnological potential, bacteria were screened for the presence of PKS (polyketide synthase) and NRPS (non-ribosomal peptide synthetases) genes. PKS genes were detected in 41 strains (25%) and NRPS genes in 73 (43%) strains by PCR analysis. The occurrence of PKS genes in members of the phylum *Firmicutes* (the genera *Bacillus* and *Paenibacillus*) was 34% and NRPS genes were found in 78%. In *Proteobacteria*, PKS and NRPS genes were found in 20% and 32%, and in 22% and 22% of *Actinobacteria*, respectively. For further analysis of PKS and NRPS genes, six *Bacillus* and *Paenibacillus* strains with antagonistic activity were selected and underwent phylogenetic analysis of 16S rRNA genes. The identification of PKS and NRPS genes in the strains investigated was demonstrated among the homologues the genes involved in the biosynthesis of antibiotics (bacillaene, difficidine, erythromycin, bacitracin, tridecaptin, and fusaricidin), biosurfactants (iturin, bacillomycin, plipastatin, fengycin, and surfactin) and antitumor agents (epothilone, calyculin, and briostatin). *Bacillus* spp. 9A and 2A strains showed the highest diversity of PKS and NRPS genes. *Bacillus* and *Paenibacillus* strains isolated from epilithic biofilms in Lake Baikal are potential producers of antimicrobial compounds and may be of practical interest for biotechnological purposes.

Key words: polyketide synthase genes, non-ribosomal peptide synthetases, epilithic biofilms, Lake Baikal, secondary metabolites

## Introduction

Microorganisms from various ecological niches are the most important source of antibiotic substances and other bioactive metabolites (Sponga et al. 1999; Lorentz et al. 2006; Wu et al. 2011; Mondol et al. 2013; Palomo et al. 2013). To date, 95–99% of microorganisms in natural biotopes exist in the form of biofilms, since this facilitates access to nutrients, promotes cooperation between microorganisms, and protects cells from negative environmental effects (Costerton et al. 1987). Biofilm is a microbially derived sessile community characterized by the cells that are irreversibly attached to a substratum, interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced, and it exhibits an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton 2002). Biofilms are a type of microbial consortia that play an important role in biogeochemical processes in the biosphere.

In the aqueous environment, biofilms exist in several types, depending on the substrate on which they are formed: epilithic (rock surfaces), epipsammic (attached to sediment particles), epixylic (on dead plant material), epiphytic (on living plants), marine or lake snow (on organic and inorganic particles), and biofouling (artificial surfaces) (Romaní et al. 2016). Compared to other biofilms, epilithic biofilms have a more complex heterogeneous structure with a higher algal biomass and a large repertory; they are also more independent of seasonal fluctuations (Romaní and Sabater 2001; Bartrons et al. 2012). Obviously, the search for the biologically active substances (BAS) among the bacteria inhabiting epilithic biofilms is promising.

Multidomain enzymatic 'megasynthases', including PKS, NRPS and their NRPS/PKS hybrid complexes,

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synthesise a wide range of secondary metabolites of the bacterial origin (Staunton and Wilkinson 2001). A diverse chemical structure and functional activity characterize polyketides, among which there are antibiotics, statins, tumor growth inhibitors, and other pharmaceutically significant compounds. There are three types of PKS (I, II, and III), which differ depending on the structure and mechanism of catalysis. Type I PKS are organized into modules consisting of at least three functional domains: ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). Each module is responsible for one elongation cycle of the polyketide chain. Type II PKS are a large multienzyme complex of small, discrete enzymes with particular functions. The pivotal component that is responsible for the condensing activity resembles $\beta$-ketoacyl synthase II of type II FAS found in bacteria and plants. This class of PKS is responsible for the biosynthesis of bacterial aromatic polyketides, such as oxytetracycline and pradimicin. Type III PKS are self-contained enzymes that form homodimers. Their single active site in each monomer catalyzes the priming, extension, and cyclization reactions iteratively to form polyketide products. Despite their structural simplicity, type III PKS produce a wide array of compounds such as chalcones, pyrones, acridones, phloroglucinols, stilbenes, and resorcinolic lipids (Dayu et al. 2012). NRPS synthesize a few natural compounds with a wide range of biological activity and various medicinal properties. Monomers of amino acids serve as substrates for the synthesis of NRPS peptides. The modules contain an ATP-dependent adenylation domain (A-domain), a peptidyl carrier protein (PCP) domain, and a condensation (C) domain. The assembled molecule is released from the enzyme complex through a thioesterase (TE) domain. The A-domain is the most conservative (Staunton and Wilkinson 2001).

The natural products obtained by these biosynthetic pathways have been widely described for cultured and uncultured strains (Wu et al. 2011; Fickers 2012). Molecular methods have been successfully used to detect and identify target genes in the organism as indicators of the production of novel secondary metabolites (Banskota et al. 2006; Palomo et al. 2013). There have been multiple studies on secondary metabolites synthesized by PKS and NRPS gene clusters in members of the phylum Firmicutes (Lorentz et al. 2006; Wu et al. 2011; Fickers 2012; Mondol et al. 2013; Zhang et al. 2013). Natural strains of the genera *Bacillus* and *Paenibacillus* have in their genomes the clusters of genes responsible for the synthesis of several active compounds (antibiotics and biosurfactants), which act synergistically, thus showing high antagonistic activity against various pathogens (Ongena and Jacques 2008; Chen et al. 2009; Kim et al. 2010; Li et al. 2012). Therefore, natural isolates of bacilli represent a rich source of new antimicrobial substances of great importance for biotechnology.

Lake Baikal, one of the largest (area of 31 722 km$^2$) and the deepest (1637 m) freshwater reservoir in the world, has a significant biodiversity and high endemism of hydrobionts, unique ecological peculiarities, and rich biotopes. It is a kind of natural laboratory for studying the metabolic potential of microbial communities. Its littoral zone occupies 7% of the total area; the coastline is 2000 km.

Previously, strains of the genera *Streptomyces* and *Micromonospora* were isolated from water, sponges, and sediments in Lake Baikal. They showed antagonistic activity against potentially pathogenic microorganisms resistant to a number of antibiotics (Terkina et al. 2006). The authors suggested that Baikal actinomycetes can be used as producers of new BAS. Quite recently, polyketide synthase genes were identified in the metagenome community of the endemic sponges *Lubomirskia baicalensis* and *Swartschewskia papyracea*. Among the closest relatives, there were the genes involved in biosynthesis of metabolites, curacin A, stigmatellin, and nostophycin (Kaluzhnaya et al. 2012; Kaluzhnaya and Itskovich 2016). In the genome of the Baikal strain *Pseudomonas fluorescens* 28Bb-06, PKS genes were identified as 50–66% homologous to the gene clusters involved in biosynthesis of yersiniabactin, rhizoxin, disorazol, and epothilone (Lipko et al. 2012). In strains isolated from the freshwater sponge *L. baicalensis*, PKS and NRPS genes were detected in nine out of 14 cultures of the genera *Bacillus*, *Pseudomonas*, *Variovorax*, *Curtobacterium*, and *Rhodococcus* (Kalyuzhnaya et al. 2013).

The formation of hydrobiont communities on various geological rocks has been studied in Lake Baikal since 2000 (Timoshkin et al. 2003). These studies showed that the development and activity of organisms depended on the chemical composition of the rocks and their structure. They also showed the high selectivity of these organisms in terms of the occupation of different substrates (Parfenova et al. 2008). For the first time, bacterial communities of water and biofilms formed on a solid substrate in Lake Baikal were studied by pyrosequencing of the 16S rRNA gene fragment. Bacterial communities of biofilms showed high taxonomic diversity, represented by *Cyanobacteria*, *Bacteroidetes*, and *Proteobacteria*; the contribution of other groups did not exceed 1% (Parfenova et al. 2013).

The genomes of bacteria *Serratia*, *Pseudomonas*, *Rheinheimera*, and *Flavobacterium* isolated from epilithic biofilms in Lake Baikal showed diversity in their PKS genes, which are responsible for the synthesis of antibiotics and cytostatics (Sukhanova et al. 2017). Previously, we determined the antimicrobial activity of *Bacillus* and *Paenibacillus* strains isolated from biofilms (Zimens et al. 2014).

This work aimed to detect and evaluate of diversity of the PKS and NRPS genes in the genomes of heterotrophic bacteria isolated from epilithic biofilms in Lake Baikal.

## Experimental

### Materials and Methods

**Sampling.** Samples of epilithic biofilms were taken from the littoral zone of Lake Baikal near the settlement of Listvyanka (Cape Beryozovy, 51°50′41.04″, 104°54′05.82″). Biofilms were sampled from plates (rocks and minerals) with a thickness of 0.5–1 cm that had been prepared in advance and were immersed in 2011 by divers at a depth of 7–8 m and exposed under natural conditions of the lake during the year. In May 2012, the plates covered with biofilms were lifted from the bottom of the lake, put in sterile containers with Baikal water and then transported to the laboratory at a temperature of 10°C. Under aseptic conditions, fouling with an area of 2 $cm^2$ was scraped, which was used for cultivation in nutrient media.

**Isolation of heterotrophic bacteria.** The samples of biofilms were suspended in 50 ml of sterile Baikal water and shaken for 30 min on a shaker at 120 rpm. A 1 ml aliquot was added to 100 ml of sterile Baikal water, then 1 ml of the resulting suspension was plated in three replicates using the pour plate method onto solid nutrient media with different contents of organic matter. To isolate pure cultures, the following nutrient media were used: R2A (Fluka analytical, USA), NSY (g/l: nutrient broth 1, soy peptone 1, yeast extract 1 and agar 15), PCA (HiMedia, India), and TSA (HiMedia, India). The duration of incubation was 5–7 days at 20–22°C. Pure cultures were obtained by depleting inoculations to individual colonies.

The average number of colonies obtained during cultivation on each medium was: R2A – 5.6, NSY – 4.9, PCA – 3.3 and TSA – 1.9 ($\times 10^5$ CFU/$cm^2$). Bacterial colonies with differing morphology were isolated into pure cultures on the nutrient media: R2A – 64 strains, PCA – 48 strains, NSY – 35 strains and TSA – 20 strains. As a result, we obtained a collection of heterotrophic bacteria of 167 strains.

**Molecular genetic identification of strains by the 16S rRNA gene fragment.** DNA from the day-old bacterial cultures was isolated using the DNA-sorb-B kit according to the manufacturer's protocol (PE CRIE of Rospotrebnadzor, Moscow, Russia). The obtained template was used in the polymerase chain reaction (PCR); target amplicons of the 16S rRNA gene fragment were obtained using the conservative bacterial primers 27L (5'-AGAGTTTGATCATGGCTCAG-3') and 1542R (5'-AAGGAGGTGATCCAGCCS-3') (Brosius et al. 1981). The nucleotide sequences of the 16S rRNA gene fragments were determined on an ABI PRISM 310A Genetic Analyser automatic sequencer (Perkin Elmer, USA) at the SB RAS Genomics Core Facility (Novosibirsk). Comparative analysis of the sequences obtained with previously published ones was carried out using the FASTA and BLAST software package. Nucleotide sequences of 167 strains were registered in GenBank under the following numbers: HF548373 – HF548383, HF548386 – HF548401, HF678874 – HF678892, HF678894 – HF678990, HF947322 – HF947328, LT555292, and LT601385 – LT601400 (personal results; unpublished data).

The numbers of the strains used in this study were as follows: *Paenibacillus* sp. 5A (HF678944), *Paenibacillus* sp. 12A (HF678945), *Paenibacillus* sp. 7A (HF678946), *Bacillus* sp. 2B (HF678932), *Bacillus* sp. 2A (HF678933), and *Bacillus* sp. 9A (HF678934).

**PCR screening of PKS and NRPS genes in the genomes of heterotrophic bacteria.** Fragments of the KS-domains of PKS genes were amplified using the degenerate primers DK-F (5'-GTGCCGGTNC-CRTGNGYYTC-3') and DK-R (5'-GCGATGGAY-CCNCARCARYG-3'); the fragment length was 700 bp (Ehrenreich et al. 2005). PKS genes were amplified under the following conditions: polymerase activation (5 min at 94°C); 35 cycles, including DNA denaturation (45 s at 94°C), primer annealing (50 s at 60°C) and elongation (60 s at 72°C), as well as final elongation (10 min at 72°C). To screen the A-domain of NRPS genes, we used the primers MT-F (5'-GCNGGYGGYGCN-TAYGTNCC-3') and MT-R (5'-CCNCGDATYTTNA-CYTG-3') (1000 bp); the PCR conditions were the same as those described above (Ehrenreich et al. 2005). PCR products were visualized on 1% agarose gel.

**Study of enzymatic activity in members of the genera *Bacillus* and *Paenibacillus*.** The ability of the strains studied to utilize carbon compounds (Hiss medium) and organic nitrogen-containing substances (amino acids) was assayed. The proteolytic extracellular enzymes were defined on media with casein and gelatine, lipolytic enzymes with tributyrin and lecithin, and amylolytic enzymes with starch (Netrusov 2005). Phosphatase activity was detected using the Alkaline Phosphatase-VITAL kit (Vital Development Corporation, Russia).

**Phylogenetic analysis of the 16S rRNA gene sequences from *Bacillus* and *Paenibacillus*.** For the species identification of *Bacillus* and *Paenibacillus* isolates, the sequences were aligned in the Clustal-W program. Phylogenetic analysis of nucleotide sequences of the 16S rRNA gene (length of 1360 bp) was carried out using the Mega 6.06 program, the Maximum Likelihood method, and the Kimura 2-parameter model. Bootstrap support was computed for 1000 replicates.

**Identification of PKS and NRPS genes in the genomes of *Bacillus* and *Paenibacillus*.** Amplicons of the gene fragments were visualized in 1% agarose gel using a transilluminator (VL-6.MC, France). The PCR fragments were cloned in the vector pJET1.2/

blunt (CloneJET PCR Cloning Kit, Fermentas, Lithuania), then amplicons were transformed in the cells of competent *E. coli* DH-5α and XL-1 strains.

Nucleotide sequences were determined on a genetic analyzer (Applied Biosystems, USA) in Irkutsk (Russia) and at the research and production company Sintol (Moscow, Russia). To transfer nucleotide sequences of the PKS and NRPS into amino acids, we used the BioEdit 7.2.5. program. A comparative analysis of the sequences obtained was carried out using the BLASTX and BLASTP software package.

Nucleotide sequences were deposited in GenBank under the numbers LT555240-LT555282 for PKS genes (43 pcs.) and LT990671-LT990687 for NRPS genes (17 pcs.).

**Phylogenetic analysis of amino acid sequences of the KS-domain fragments of PKS genes and A-domain of NRPS genes** was carried out using the Mega 6.06 program, the Neighbor-joining method, and the Kimura 2-parameter model. Bootstrap support was computed for 1000 replicates. The sequences were aligned in the Clustal-W program.

Table I
PCR screening of heterotrophic strains isolated from biofilms of Lake Baikal for PKS and NRPS gene fragments.

| Taxonomy | | Number of the strains analyzed | Number of the strains with positive PCR signal | |
|---|---|---|---|---|
| Phylum | Genus | | PKS | NRPS |
| *Firmicutes* | *Bacillus* | 42 | 11 | 33 |
| | *Paenibacillus* | 4 | 4 | 4 |
| | *Virgibacillus* | 1 | 0 | 0 |
| | *Staphylococcus* | 2 | 0 | 1 |
| *Proteobacteria* | *Pseudomonas* | 45 | 9 | 26 |
| | *Aeromonas* | 29 | 4 | 2 |
| | *Serratia* | 3 | 2 | 1 |
| | *Rhizobium* | 1 | 1 | 0 |
| | *Brevundimonas* | 1 | 0 | 0 |
| | *Massilia* | 1 | 0 | 0 |
| | *Achromobacter* | 3 | 0 | 0 |
| | *Stenotrophomonas* | 3 | 0 | 0 |
| | *Devosia* | 1 | 1 | 0 |
| | *Hydrogenophaga* | 1 | 0 | 0 |
| | *Yersinia* | 1 | 1 | 0 |
| | *Sphingomonas* | 1 | 0 | 0 |
| | *Iodobacter* | 1 | 1 | 0 |
| | *Roseomonas* | 1 | 0 | 0 |
| *Actinobacteria* | *Rhodococcus* | 2 | 0 | 2 |
| | *Kocuria* | 4 | 2 | 1 |
| | *Pseudoclavibacter* | 3 | 1 | 0 |
| | *Plantibacter* | 1 | 0 | 0 |
| | *Sanguibacter* | 1 | 0 | 0 |
| | *Pseudarthrobacter* | 1 | 0 | 0 |
| | *Microbacterium* | 4 | 1 | 1 |
| | *Salinibacterium* | 1 | 0 | 0 |
| | *Streptomyces* | 1 | 0 | 1 |
| | *Micrococcus* | 1 | 0 | 0 |
| | *Brachybacterium* | 1 | 1 | 0 |
| | *Clachiihabitans* | 1 | 0 | 0 |
| | *Microcella* | 1 | 0 | 0 |
| *Bacteroidetes* | *Flavobacterium* | 4 | 2 | 1 |
| Total | | 167 | 41 | 73 |

## Results

Table I shows the results of strain isolation from epilithic biofilms in Lake Baikal. We obtained a collection of heterotrophic bacteria consisting of 167 strains. The isolates classified by a comparative analysis of the 16S rRNA gene fragment belonged to four phyla: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* and 32 genera of bacteria. The members of the genera *Aeromonas, Pseudomonas*, and *Bacillus* were the dominant strains (Table I).

**PCR screening of the isolates for the presence of PKS and NRPS genes.** Screening of PKS genes in the genomes of heterotrophic bacteria revealed their presence in 41 strains belonging to 14 genera: *Bacillus, Paenibacillus*, *Pseudomonas*, *Aeromonas*, *Serratia*, *Rhizobium*, *Devosia*, *Yersinia*, *Iodobacter*, *Kocuria*, *Pseudoclavibacter*, *Microbacterium*, *Brachybacterium*, and *Flavobacterium* (Table I). The total percentage of the strains with PKS genes was 25%. The occurrence of PKS genes in members of the phylum *Firmicutes* (*Bacillus, Paenibacillus*) was 34%, 20% in *Proteobacteria* and 22% in *Actinobacteria* (Table I).

The screening of 167 strains showed a positive PCR signal for the presence of NRPS genes in 73 strains of 11 genera: *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Pseudomonas*, *Aeromonas*, *Serratia*, *Rhodococcus*, *Kocuria*, *Microbacterium*, *Streptomyces*, and *Flavobacterium* (Table I). The total percentage of strains containing NRPS genes was 43%. A high percentage of these genes was found in the genus *Pseudomonas* (57%). At the same time, the occurrence of NRPS genes in members of the phylum *Firmicutes* (*Bacillus* and *Paenibacillus*) reached 78% of the total number of strains from this group. These genes were found in 32% of *Proteobacteria* and 22% of *Actinobacteria* (Table I).

The phylogenetic diversity of the genera *Bacillus* and *Paenibacillus* isolated from epilithic biofilms in Lake Baikal and the presence of PKS and NRPS genes in their genomes are shown in Fig. 1.

Thus, NRPS genes were more commonly found in *Bacillus* and *Pseudomonas*; the members of the phyla *Firmicutes* (*Bacillus, Paenibacillus*) also had high percentage of PCR positive strains with both, PKS and NRPS genes.

**Physiological and biochemical characteristics of *Bacillus* and *Paenibacillus* strains.** At the next stage, based on the obtained results of PCR screening, we selected six cultures: *Paenibacillus* spp. 5A, 12A, and 7A and *Bacillus* spp. 2A, 2B, and 9A. Previously, these strains showed antagonistic activity (Table II) (Zimens et al. 2014). Among them, there were highly active *Paenibacillus* spp. 5A and 12A and *Bacillus* sp. 9A, which simultaneously suppressed the growth of test cultures from different taxonomic groups (Gram-positive and Gram-negative bacteria, as well as fungi). Hence, we can assume that the strains studied can produce several different antimicrobial compounds (Zimens et al. 2014).

The selected isolates were tested for the ability to produce extracellular enzymes (Table III). We found that *Paenibacillus* spp. strains most actively utilized carbohydrates and polyatomic alcohols, and *Bacillus* spp. strains used amino acids. All cultures showed the ability to utilize starch and casein (Table III). The data on the physiological and biochemical characteristics of *Paenibacillus* spp. 5A and 12A and *Bacillus* sp. 2A and 9A were consistent with the data from the phylogenetic analysis.

**Phylogenetic analysis of the nucleotide sequences of the 16S rRNA gene from *Bacillus* and *Paenibacillus* strains.** Phylogenetic analysis indicated that the nucleotide sequences of the 16S rRNA gene of *Paenibacillus* spp. 5A and 12A strains formed a separate sister cluster with the type strain *Paenibacillus peoriae* KCTC 3763$^T$ (Fig. 2). This strain, isolated from soil, is antagonistic against phytopathogenic bacteria and fungi (Jeong

Table II
Antagonist activity (Zimens et al. 2014), and PKS and NRPS genes in the strains isolated from biofilms of Lake Baikal.

| Strain | Antagonist activity | PCR signal to PKS gene | PCR signal to NRPS gene |
|---|---|---|---|
| *Paenibacillus* sp. 5A | Ec1*, Ec2, Pa, Bs1, Bs2, Ca, Sa, Ef | + | + |
| *Paenibacillus* sp. 12A | Ec1, Ec2, Pa, Bs1, Bs2, Ca, Sa, Ef | + | + |
| *Paenibacillus* sp. 7A | Bs1, Bs2, Sa, Ca | + | + |
| *Bacillus* sp. 2A | Ec1, Bs1, Bs2, Ca | + | + |
| *Bacillus* sp. 9A | Bs1, Bs2, Ca, Sa, Ef | + | + |
| *Bacillus* sp. 2B | Ec1, Bs2 | + | + |

* Test cultures used in this work: Bs1 – *Bacillus subtilis* VKPM; Bs2 – *Bacillus subtilis* DSM; Pa – *Pseudomonas aeruginosa* GISK L.A. Tarasevich 190158; Ca – *Candida albicans* ATCC 10231; Sa – *Staphylococcus aureus* (ATCC 25923 and MRSA); Ef – *Enterococcus faecium*; Ec1 – *Escherichia coli* K12 VKPM B-3254; Ec2 – *Escherichia coli* M17-02 VKPM B-8208

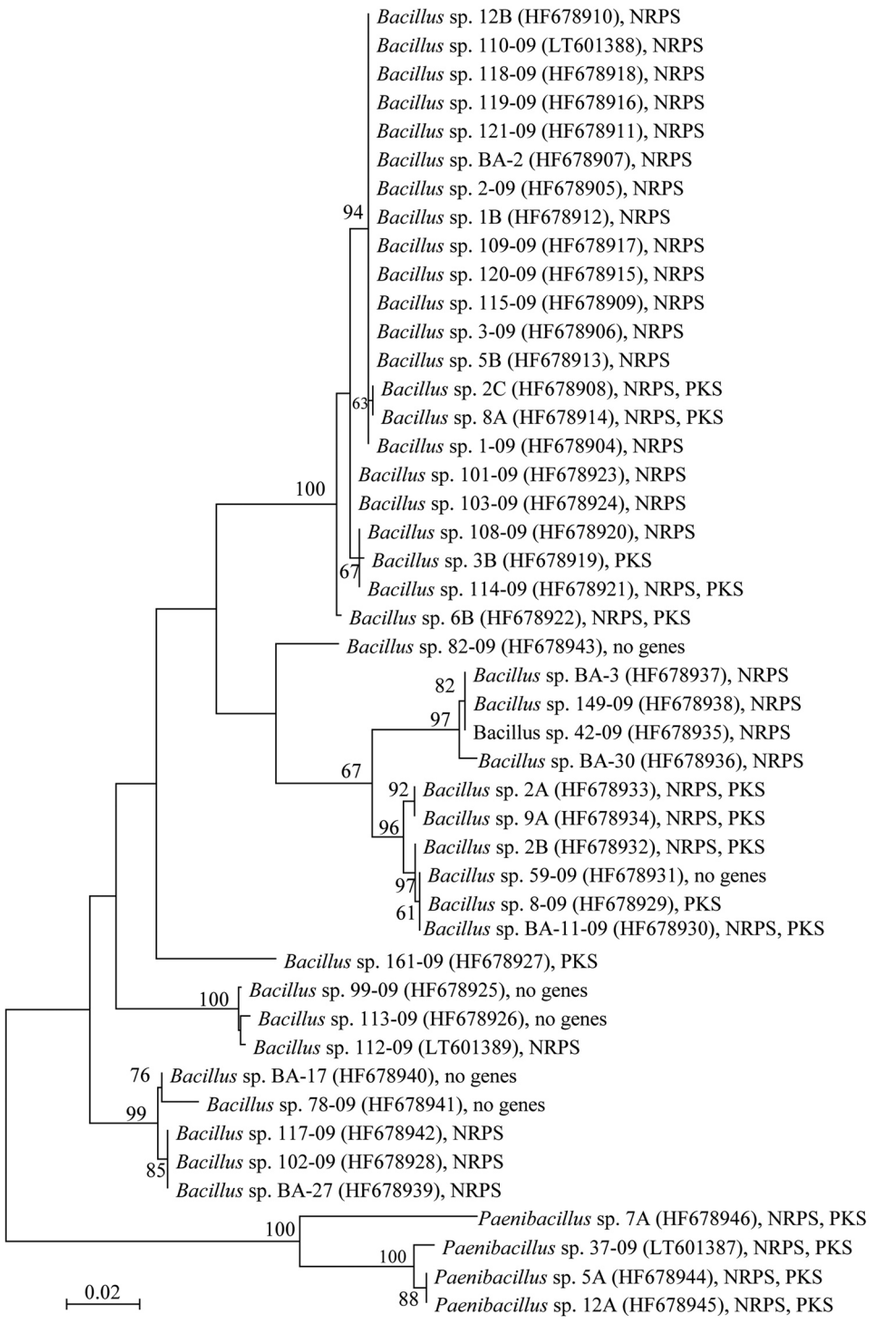


Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences (880 bp), showing the phylogenetic relationship between strains of the genera *Bacillus* and *Paenibacillus* isolated from epilithic biofilms in Lake Baikal. Accession numbers in GenBank are given in parentheses.

et al. 2012). On the phylogenetic tree, the nucleotide sequence from *Paenibacillus* sp. 7A clusters with the sequence of the nitrogen-fixing bacterium *Paenibacillus graminis* RSA19$^T$ isolated from the maize rhizosphere (Berge et al. 2002), which allowed us to preliminarily classify this strain as *P. graminis* 7A.

Nucleotide sequences of the 16S rRNA gene from *Bacillus* spp. 2A and 9A strains formed a joint cluster with the type strain *Bacillus amyloliquefaciens* NBRC 15535$^T$ (Fig. 2) isolated from fermented locust bean fruits (Africa) (Meerak et al. 2008). Strains 2A and 9A were preliminarily assigned to the species *B. amyloliquefaciens*.

Phylogenetic analysis indicated that the nucleotide sequence of the 16S rRNA gene from *Bacillus* sp. 2B clustered with the type strain *Bacillus subtilis* DSM10$^T$

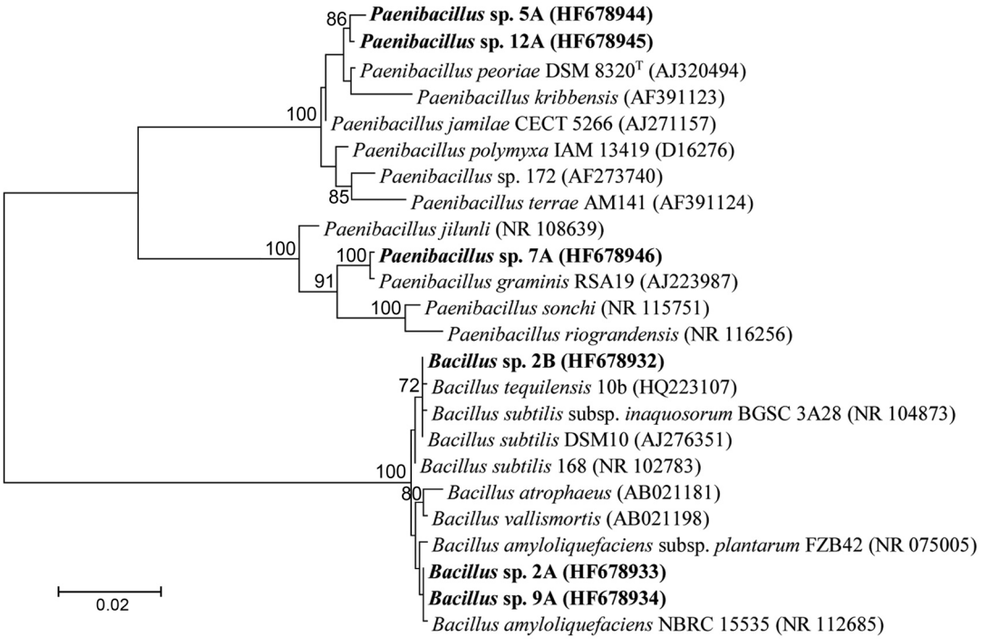


Fig. 2. Phylogenetic tree, based on 16S rRNA gene sequences (1360 bp), showing the phylogenetic relationship between the strains studied and other species of the genera *Bacillus* and *Paenibacillus*.
Sequences obtained in this study are in bold, and accession numbers in GenBank are given in parentheses.

(Fig. 2). Additionally, it showed high homology (100%), thus we attributed the *Bacillus* sp. 2B strain to the species *B. subtilis*.

**Identification of PKS genes in the genomes of *Bacillus* and *Paenibacillus* strains.** Molecular genetic analysis indicated 43 nucleotide sequences that were 96–100% similar to published sequences. All identified gene fragments were assigned to the modular type I PKS. Table IV shows the closest homologues determined by the BLAST-analysis and Table V presents the polyketide synthase genes identified.

We determined eight nucleotide sequences of PKS genes for both *Paenibacillus* spp. 5A and 12A (Table IV); the closest homologues were obtained from *Paenibacillus polymyxa* and *P. peoriae*. Among the homologous sequences obtained from the 12A and 5A strains, there were genes for the synthesis of antibiotics (difficidine, erythromycin, bacillaene, batumin, and

Table III
The strain screening for the presence of enzyme activity.

| Characteristic | *Paenibacillus* spp. | | | *Bacillus* spp. | | |
|---|---|---|---|---|---|---|
| | 5A | 12A | 7A | 2A | 9A | 2B |
| Phosphatase | – | – | – | + | + | + |
| Catalase | + | + | + | + | + | + |
| Oxidase | – | + | – | – | – | – |
| Gelatinase | – | – | – | + | + | – |
| Caseinase | + | + | + | + | + | + |
| Amylase | + | + | + | + | + | + |
| Lecithinase | + | + | – | + | + | + |
| Lipase | – | – | – | – | – | – |
| Saccharose | + | + | + | – | – | + |
| Glucose | + | + | + | – | – | + |
| Maltose | + | + | + | – | – | – |
| Fructose | + | + | +g* | – | – | + |
| Galactose | +g | +g | + | – | – | – |
| Lactose | + | + | +g | – | – | – |

Table III
Continued.

| Characteristic | *Paenibacillus* spp. | | | *Bacillus* spp. | | |
|---|---|---|---|---|---|---|
| | 5A | 12A | 7A | 2A | 9A | 2B |
| Arabinose | + | + | + | – | – | – |
| Raffinose | + | + | + | – | – | – |
| Rhamnose | +g | +g | + | – | – | – |
| Mannose | + | + | + | – | – | + |
| Xylose | + | + | + | – | – | – |
| Sorbitol | + | + | + | – | – | – |
| Inositol | + | + | + | – | – | + |
| Dulcite | +g | +g | + | – | – | – |
| Mannitol | +g | +g | + | – | – | – |
| Proline | – | – | + | + | + | + |
| Leucine | – | – | – | + | + | + |
| Phenylalanine | – | – | – | + | + | + |
| Alanine | – | – | – | + | + | + |

*+g – sugar fermentation together with the formation of acid and gas.

Table IV
Comparative analysis of the sequences of PKS gene fragments from heterotrophic bacteria.

| Strain name | Clone number | Results of BLAST analysis | |
|---|---|---|---|
| | | Closest homologs | Homology, % |
| *Paenibacillus* spp. 5A and 12A | 5A-1, 5A-2, 5A-3, 5A-4, 5A-7, 5A-8, 12A-2, 12A-9 | ACN13122 ketosynthase [*Streptomyces* sp. G2-4] | 99 |
| | | WP_053325747 polyketide synthase [*P. peoriae*] | |
| | | WP_013310977 polyketide synthase [*P. polymyxa*] | 98–100 |
| | 5A-5,12A-1, 12A-5, 12A-6 | ACX31707 ketosynthase [*Streptomyces* sp. 28HAO] | 99 |
| | | WP_013310977 polyketide synthase [*P. polymyxa*] | 99 |
| | 5A-6 | WP_013310977 polyketide synthase [*P. polymyxa*] | 97 |
| | | WP_053325747 polyketide synthase [*P. peoriae*] | 97 |
| | 12A-7, 12A-10 | WP_053325746 polyketide synthase [*P. peoriae*] | 97 |
| | | WP_023989388 polyketide synthase [*P. polymyxa*] | 97 |
| | 12A-8 | WP_053325746 polyketide synthase [*P. peoriae*] | 96 |
| | | WP_023989388 polyketide synthase [*P. polymyxa*] | 96 |
| *Paenibacillus* sp.7A | 7A-1, 7A-2, 7A-5, 7A-7 7A-3, 7A-4, 7A-6 7A-8 | AIQ67612 erythronolid synthase [*P. graminis*] | 96–98 |
| | | WP_042266339 NRPS/PKS-synthase [*P. graminis*] | 98–99 |
| | | WP_042266418 polyketide synthase [*P. graminis*] | 99 |
| *Bacillus* spp. 2A and 2B | 2A-1, 2A-3, 2A-4, 2A-6, 2A-7, 2A-8, 2B-3, 2B-4 | ABR19768 polyketide synthase [*B. subtilis*] | 98–99 |
| | 2A-2 | ABR19764 polyketide synthase [*Bacillus subtilis*] | 98 |
| | | ABR19779 polyketide synthase [*Actinomycetales bacterium* DA20] | 98 |
| | 2B-1 | WP_032721576 polyketide synthase [*B. subtilis*] | 97 |
| | | AGA23985 NRPS/PKS-synthase [*B. subtilis* subsp. *subtilis* BSP1] | 97 |
| | 2B-2 | ABR19775 polyketide synthase [*B. subtilis*] | 98 |
| | 2B-5 | WP_043940121 polyketide synthase [*Bacillus* sp. YP1] | 98 |
| | | WP_009967299 polyketide synthase [*B. subtilis*] | 98 |
| | 2B-6, 2B-8 | ACG70843 polyketide synthase [*Bacillus* sp. WPhG3] | 99 |
| | 2B-7 | ABR19767 polyketide synthase [*B. subtilis*] | 98 |
| *Bacillus* sp. 9A | 9A-1 | AIO09652 ketosynthase [*Bacillus* sp. LX-110] | 99 |
| | 9A-3, 9A-5 | ACG70841 polyketide synthase [*Bacillus* sp. WPySW2] | 98 |
| | | AGL92430 polyketide synthase [*B. amyloliquefaciens*] | 99 |
| | 9A-10 | WP_016936042 polyketide synthase [*B. siamensis*] | 99 |
| | | WP_047474891 polyketide synthase [*B. amyloliquefaciens*] | 99 |

sorangicin) and antitumor agents (calyculin and bryostatin) (Table V).

We identified eight nucleotide sequences of polyketide synthases for *Paenibacillus* sp. 7A strain (Table IV). Comparative analysis indicated that all the closest homologues were obtained from *P. graminis*. Among them, we detected erythronolide synthase with high homology (97%). In addition, PKS sequences of *Paenibacillus* sp. 7A differed in structure from genes isolated from the *Paenibacillus* spp. 5A and 12A strains. Among the similar sequences, there were synthases of the antibiotic plipastatine and the antitumor agent epothilone with 43–53% homology (Table V).

In the genomes of *Bacillus* spp. 2A and 2B strains, we identified seven and eight nucleotide sequences of the PKS gene fragment, respectively (Table IV). Interestingly, PKS sequences obtained from *Bacillus* strains had the closest relatives isolated from marine sponges (Zhang et al. 2009). Moreover, we detected genes of enzymes involved in the production of antibiotics (bacillaene and difficidine) and an antitumor agent (calyculin); homology was 70–87% (Table V).

For *Bacillus* sp. 9A strain, we detected four PKS sequences (Table IV). They were homologous to the PKS genes obtained from strains of the genus *Bacillus* spp., including the species *B. amyloliquefaciens*. Additionally, among the related sequences, there were PKS genes with high homology (94–98%) that were responsible for the synthesis of antibiotics (difficidine and bacillaene) (Table V).

**Identification of NRPS genes in the genomes of *Bacillus* and *Paenibacillus* strains.** Molecular genetic

Table V
Comparative analysis of the sequences of PKS genes from heterotrophic bacteria.

| Strain | Clone number | Results of BLAST analysis | |
|---|---|---|---|
| | | Homologs with identified protein | Homology, % |
| *Paenibacillus* 5A and 12A | 5A-1, 5A-2, 5A-3, 5A-4, 5A-7, 5A-8, 12A-2, 12A-9 | EJD67453 difficidin synthase, (*DfnD*) [*Bacillus* sp. 916] | 71 |
| | | EIF13796 difficidin synthase, (*DfnG*) [*Bacillus* sp. 5B6] | 71 |
| | 5A-5, 12A-1, 12A-5, 12A-6 | BAP05593 calyculin synthase, (CalE), uncultured [*Entotheonella* sp.] | 71 |
| | | ADN68476 sorangicin synthase, (SorA) [*Sorangium cellulosum* So ce12] | 71 |
| | 5A-6 | WP_004619353 erythronolid synthase, [*Clostridium papyrosolvens* DSM 2782] | 75 |
| | | EIF13279 bacillaene synthase, (*BaeL*) [*Bacillus* sp. 5B6] | 73 |
| | 12A-7, 12A-10 | ADD82940 batumin synthase, (*Bat2*) [*P. fluorescens* BCCM_ID9359] | 70 |
| | | ABK51300 bryostatin synthase, (*BryC*) [*Endobugula sertula*] | 69 |
| | 12A-8 | EIF13279 bacillaene synthase, (*BaeL*) [*Bacillus* sp. 5B6] | 72 |
| | | ADN68477 sorangicin synthase, (*SorB*) [*Sorangium cellulosum* So ce12] | 72 |
| *Paenibacillus* sp.7A | 7A-1, 7A-2, 7A-5, 7A-7 | AIQ67612 erythronolid synthase, [*P. graminis* DSM 15220] | 97 |
| | 7A-3, 7A-4, 7A-6 | CUB31962 plipastatin synthase, [*B. amyloliquefaciens*] | 53 |
| | | WP004618786 erythronolid synthase, [*Clostridium papyrosolvens* DSM 2782] | 50 |
| | 7A-8 | WP_013663185 erythronolid synthase, [*Marinomonas mediterranea* MMB-1] | 43 |
| | | ADB12491 epothilone synthase, (*EpoD*) [*Sorangium cellulosum* KYC3013] | 43 |
| *Bacillus* spp. 2A and 2B | 2A-1, 2A-3, 2A-4, 2A-6, 2A-7, 2A-8, 2B-3, 2B-4 | EJD66458 bacillaene synthase, (*BaeN*) [*Bacillus* sp. 916] | 85 |
| | 2A-2 | BAP05593 calyculin synthase, (*CalE*), [*Entotheonella* sp.] | 71 |
| | | EJD67453 difficidin synthase, (*DfnD*) [*Bacillus* sp. 916] | 70 |
| | 2B-1 | EIF13279 bacillaene synthase, (*BaeL*) [*Bacillus* sp. 5B6] | 85 |
| | 2B-2 | EIF13280 bacillaene synthase, (*BaeM*) [*Bacillus* sp. 5B6] | 84 |
| | 2B-5 | EIF13279 bacillaene synthase, (*BaeL*) [*Bacillus* sp. 5B6] | 71 |
| | 2B-6, 2B-8 | EJD66458 bacillaene synthase, (*BaeN*) [*Bacillus* sp. 916]. | 87 |
| | 2B-7 | EIF13279 bacillaene synthase, (*BaeL*) [*Bacillus* sp. 5B6]. | 86 |
| | | AFZ90784 bacillaene synthase, (*BaeL*) [*B. methylotrophicus* AS43.3] | 86 |
| *Bacillus* sp. 9A | 9A-1 | WP_049628737 difficidin synthase, [*Bacillus* sp. JFL15] | 98 |
| | 9A-3, 9A-5 | EJD66458 bacillaene synthase, (*BaeN*) [*Bacillus* sp. 916] | 94 |
| | 9A-10 | EIF13279 bacillaene synthase, (*BaeL*) [*Bacillus* sp. 5B6] | 97 |

analysis indicated 17 nucleotide sequences of the NRPS gene fragment that were 95–100% similar to published sequences (Table VI).

For the *Paenibacillus* sp. 12A strain, we obtained five nucleotide sequences of the NRPS gene fragment which had homologues isolated from *Paenibacillus polymyxa* (Table VI). The homologous sequences included genes for the synthesis of antibiotics (bacitracin, fusaricidin, tridecaptin, and bacillorin). In *Paenibacillus* sp. 5A, two sequences of the NRPS gene fragment were detected. The homologous sequences included genes for the synthesis of antibiotics (bacitracin and fusaricidin) and low homology to fengycin (Table VI).

We identified two sequences of the NRPS gene fragment in *Bacillus* sp. 2B strain. Among the homologues, there were genes coding for the enzymes responsible for the synthesis of biosurfactants (plipastatin and suractin) (Table VI).

Three sequences of the NRPS gene fragment were determined *Bacillus* sp. 2A strain. The homologous sequences included genes for the synthesis of an antibiotic (bacillaene) and biosurfactants (plipastatin, fengycin, and surfactin) (Table VI).

We detected four sequences of the NRPS gene fragment in *Bacillus* sp. 9A strain. The homologous sequences included genes responsible for the synthesis

Table VI
Comparative analysis of the sequences of NRPS genes from heterotrophic bacteria.

| Strain | Clone number | Results of BLAST analysis | |
|---|---|---|---|
| | | Closest homologs | Homology, % |
| *Paenibacillus* spp. 5A and 12A | 5A-4 | WP_029514857 non-ribosomal peptide synthetase [*P. polymyxa*] | 95 |
| | | ADJ56349 fengycin A [*Bacillus subtilis*] | 56 |
| | 5A-5, 12A-1, 12A-2 | KOS00083 bacitracin synthetase [*P. polymyxa*] | 97–100 |
| | | ABQ96384 fusaricidin synthetase [*P. polymyxa*] | 96–99 |
| | 12A-5 | AKH45460 tridecaptin, TrbE [*P. polymyxa*] | 96 |
| | | KOS03006 fusaricidin synthetase [*P. polymyxa*] | 96 |
| | 12A-6 | WP_013310169 NRPS/PKS synthase [*P. polymyxa*] | 96 |
| | | KOS01021 bacitracin synthetase [*P. polymyxa*] | 94 |
| | | AHM66091 bacillorin synthetase b [*P. polymyxa* SQR-21] | 94 |
| | | AKH45459 tridecaptin B, TrbD [*P. polymyxa*] | 93 |
| | | KTS81295 fusaricidin synthetase [*P. jamilae*] | 92 |
| | 12A-10 | WP_025721680 non-ribosomal peptide synthetase [*P. polymyxa*] | 99 |
| | | ABQ96384 fusaricidin synthetase [*P. polymyxa*] | 96 |
| | | KOS01669 bacitracin synthetase [*P. polymyxa*] | 94 |
| *Bacillus* sp. 2B | 2B-1 | WP_080466694 plipastatin synthetase [*B. subtilis*] | 100 |
| | 2B-3 | CAA49816 surfactin synthetase, srfA [*B. subtilis*] | 99 |
| *Bacillus* sp. 2A | 2A-5 | WP_019712401 plipastatin synthetase subunit B [*B. subtilis*] | 98 |
| | | ACX47457 fengycin synthetase B [*B. subtilis*]. | 98 |
| | 2A-7 | AOA54360 polyketide synthase PksJ [*B. subtilis*] | 98 |
| | | WP_009967299 non-ribosomal peptide synthetase [*B. subtilis*] | 99 |
| | 2A-8 | WP_048654751 surfactin synthetase SrfAA [*B. subtilis*] | 97 |
| *Bacillus* sp. 9A | 9A-1 | WP_047474893 non-ribosomal peptide synthetase [*B. amyloliquefaciens*] | 99 |
| | | ASB53193 polyketide synthase PksJ [*B. velezensis*] | 94 |
| | 9A-4 | AHW81970 bacillomycin [*B. subtilis*] | 96 |
| | 9A-8 | WP_064777945 surfactin non-ribosomal peptide synthetase SrfAA [*B. siamensis*] | 98 |
| | 9A-9 | WP_045925809 non-ribosomal peptide synthetase [*B. siamensis*] | 98 |
| | | CCF05308 iturinA synthetase ItuA [*B. velezensis* CAU B946] | 93 |

of antibiotic (bacillaene) and biosurfactants (bacillomycin, surfactin, and iturin) (Table VI).

Phylogenetic analysis of amino acid sequences of the KS-domain fragments of PKS genes (Fig. 3) and A-domain of NRPS genes (Fig. 4) in the bacteria isolated from the epilithic biofilms of Lake Baikal showed that sequences from different strains clustered together. It means that enzyme complexes of such strains as *Bacillus* spp. 2A and 2B, *Paenibacillus* spp. 5A and 12A were similar. On the other hand, different sequences were obtained from one strain. It means that this strain, e.g. *Bacillus* sp. 9A, possessed several enzyme complexes.

## Discussion

The results of PCR screening showed that PKS and NRPS genes in members of *Bacillus*, *Paenibacillus*, and *Pseudomonas* from Lake Baikal were more frequent than in other heterotrophic bacteria isolated from biofilms. The high occurrence of the BAS genes found in Baikal isolates is typical of the members belonging to these genera, since they are well-known producers of various secondary metabolites. For example, many *Bacillus* species produce such antibiotics as bacillaene, difficidine, macrolactin, mycosubtilin, bacillomycin, iturin, bacitracin, and gramicidin C (Fickers 2012). *Paenibacillus* strains isolated from various habitats synthesize antibiotics of a peptide or macrolide nature: polymyxins A-E, paenibacillin, jolipeptin, gavaserin, saltavalin, fusaricidin A-D, gatavalin, paenimacrolidine, paenilamicin, and others (Wu et al. 2011; Aleti et al. 2015). A review by Zhao and Kuipers (2016) represented the analysis of 328 full genomes of 57 species of the family *Bacillaceae*, including 30 species of the genus *Bacillus* and 16 species of *Paenibacillus*. NRPS gene clusters were present in 70% of the tested genomes,

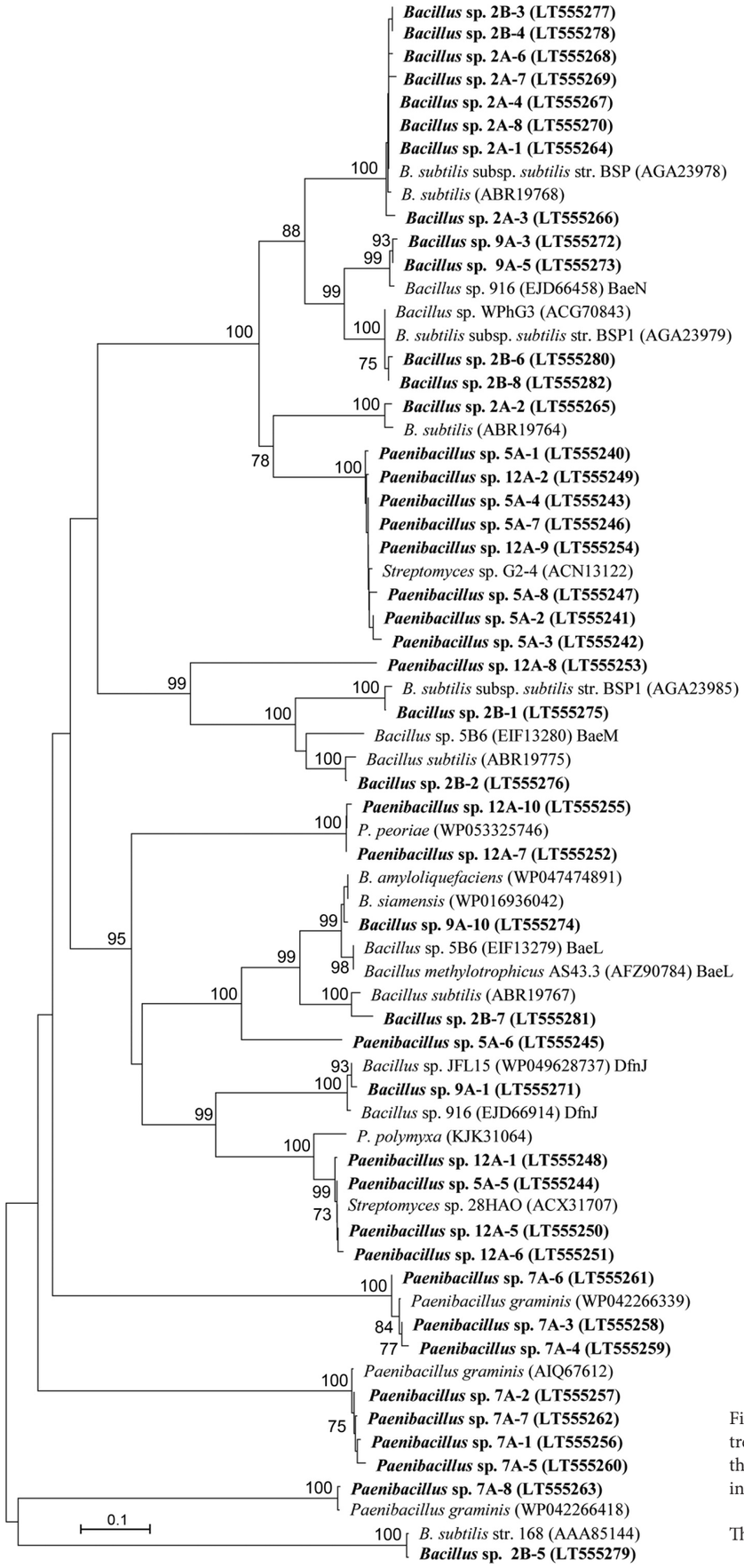


Fig. 3. Neighbour-joining phylogenetic tree based on amino acid sequences of the KS-domain fragments of PKS genes in bacteria isolated from the epilithic biofilms of Lake Baikal (in bold). The scale bar represents 0.1 amino acid substitutions per site.

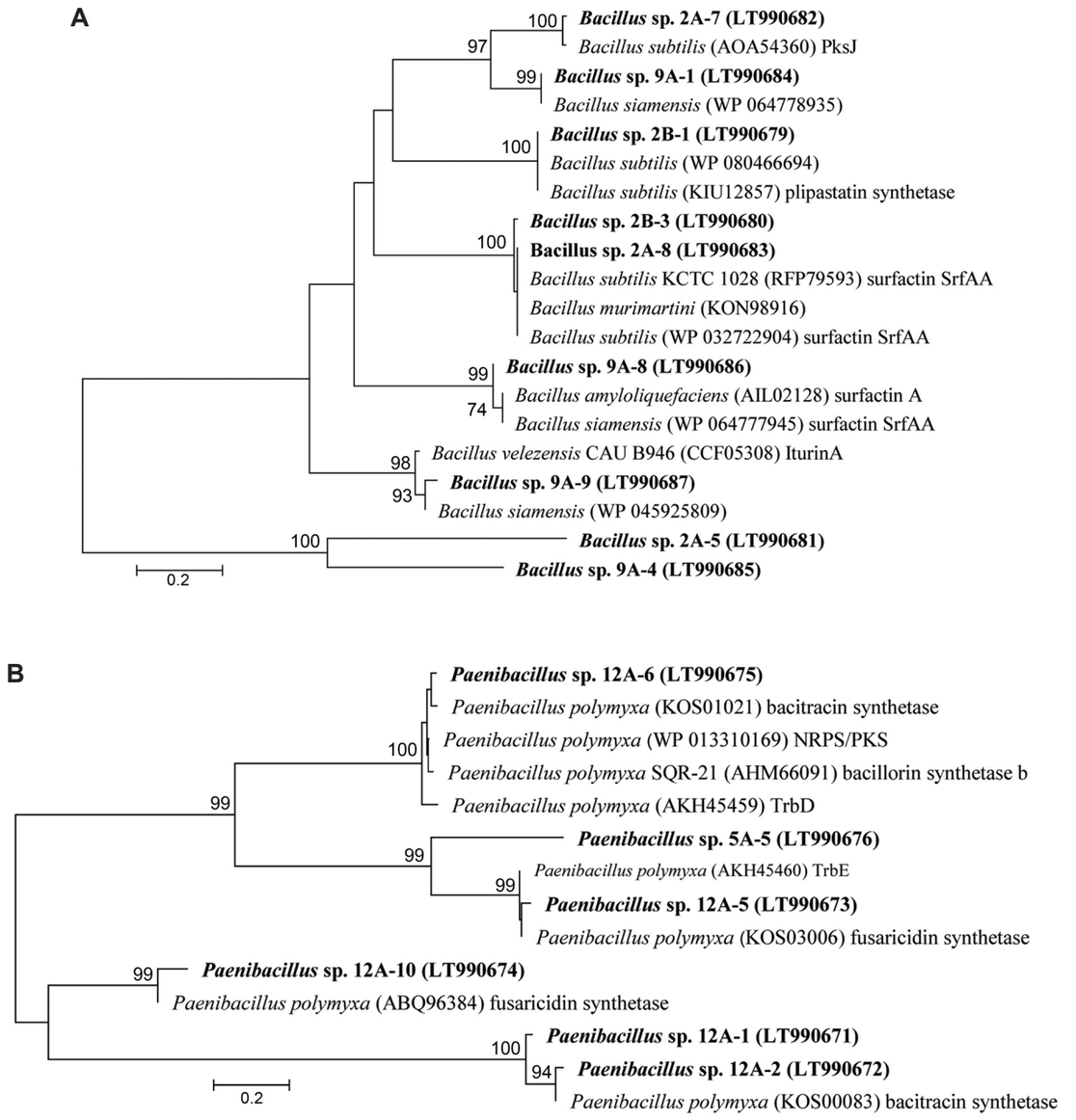


Fig. 4. Neighbour-joining phylogenetic tree based on amino acid sequences of the A-domain fragments of NRPS genes in bacteria isolated from the epilithic biofilms of Lake Baikal (in bold): a) genus *Bacillus* and b) genus *Paenibacillus*. The scale bar represents 0.2 amino acid substitutions per site.

and only 50% of the analyzed species had genes encoding PKS (Zhao and Kuipers, 2016). In total, 1231 gene clusters for putative non-ribosomal antimicrobials were identified and combined into 23 types of NRPS, five types of PKS, and three types of hybrid synthesized NRPS/PKS compounds distributed across 49 *Bacillales* species. Previously, other authors also noted the high content of NRPS and PKS genes in bacilli (Aleti et al. 2015). In addition, a high percentage of isolates (85%) containing one or both metabolic clusters were isolated from the rhizosphere (Aleti et al. 2015). The authors noted that this was due to a more detailed study of the rhizosphere as an important subject in agriculture; hence, these genes may be also characteristic of bacilli from other ecological niches. For instance, this study on *Bacillus* and *Paenibacillus* strains from freshwater reservoirs has shown that they also contain NRPS and PKS genes.

Metabolites produced by *B. amyloliquefaciens* and *B. subtilis* represent a bulk of the studied diversity of polyketides and lipopeptides from the genus *Bacillus* (Aleti et al. 2015). These two species are used to obtain most of the commercially available substances contributing to the plant growth and biocontrol (against phy-

topathogens) in agriculture. They produce three types of polyene polyketides, including bacillaene, difficidine, and macrolactin. At present, two polyketides (paenimacrolidine and paenilamicin) have been described for the genus *Paenibacillus* (Aleti et al. 2015).

In *Bacillus* and *Pseudomonas*, the NRPS genes mainly encode for the synthesis of lipopeptide biosurfactants (LPBS) (Roongsawang et al. 2010). Due to their complex and diverse structures, lipopeptides demonstrate various biological activities, including surface activity, as well as anticellular and antienzymatic activity. Lipopeptides are involved in multicellular behaviour, such as swarming motility and biofilm formation. Among the producers, the genera *Bacillus* and *Pseudomonas* are of special interest, since they produce a wide range of effective LPBS, which are potentially useful for agricultural, chemical, food, and pharmaceutical industries (Roongsawang et al. 2010). NRPS clusters of the genus *Bacillus* encode lipopeptide families of surfactin, fengycin, iturin, and kurstatin (Aleti et al. 2015).

The results of this study indicate that heterotrophic bacteria isolated from epilithic biofilms in Lake Baikal are potential producers of secondary metabolites, for which the synthesis involves PKS and NRPS gene clusters.

Identification of PKS genes has shown that *Bacillus* sp. strain 9A contains sequences in the genome that are related to the genes known for the synthesis of antibiotics bacillaene (*baeL, baeN*) and difficidine, which can indicate their ability to produce these compounds whereas *Bacillus* sp. 2A and 2B contains only bacillaene (*baeL, baeM, baeN*). Bacillaene is a polyene antibiotic and it was first found in the culture medium of *B. subtilis* 3610 and 55422 strains (Fickers 2012; Aleti et al. 2015). Its biosynthesis was described in *B. amyloliquefaciens* FZB42 and is encoded by a hybrid cluster of PKS-NRPS genes called bae. This cluster has a similar structure to the pksX cluster of *B. subtilis* 168 strain, which is also likely to encode bacillaene. The bae gene cluster contains five open reading frames, i.e. *baeJ*, *baeL*, *baeM*, *baeN*, and *baeR* (Aleti et al. 2015). Difficidine is a macrocyclic polyene synthesized by *B. amyloliquefaciens* ATCC 39320 and ATCC 39374 strains. It is encoded by the dif gene cluster with 14 open reading frames, from *difA* to *difN* and *difY*. Difficidine and bacillaene exhibit antimicrobial activity against a wide range of pathogenic bacteria by inhibiting protein synthesis (Fickers 2012; Aleti et al. 2015).

Another strain, *Paenibacillus* sp. 7A, has genes with high homology to erythronolide synthase responsible for the biosynthesis of macrolide 6-desoxy-erythronolide B, which is the precursor of the well-studied and widely known antibiotic erythromycin. It was first isolated in 1949 from the culture liquid of a *Saccharopolyspora erythraea* strain (Liu et al. 2013). The effect of this antibiotic is due to binding to the 50S ribosome subunit, which disrupts the formation of peptide links between amino acid molecules and blocks peptide synthesis in microorganisms.

Despite the high percentage of similarity (96–100%) with the closest relatives of PKS genes from *Paenibacillus* spp. 5A and 12A strains, the homologues had low similarity with the identified polyketide synthases (69–75%). It is likely that these genes have not been characterized yet, and these strains can produce novel and previously undescribed secondary metabolites.

Identification of NRPS genes showed that the sequences from *Paenibacillus* spp. 5A and 12A had high homology with their closest relatives, among which there were genes encoding for the synthesis of peptide and lipopeptide antibiotics (bacitracin, bacillorin, fusaricidin, and tridecaptin).

Bacitracin is a polypeptide antibiotic and a mixture of related cyclic peptides produced by *B. subtilis* strains. Bacitracin is active against Gram-positive bacteria. It was first isolated in 1945. It is usually used for topical treatment of skin, eye or nose diseases, but it can also be used internally in the form of an injection as an intestinal antiseptic. Due to its toxic effect on kidneys, bacitracin is used only when other antibiotics are ineffective. Its action involves breaking the synthesis of the cell wall by inhibiting lipid carriers (Johnson et al. 1945; Karala and Ruddock 2010; Ciesiołka et al. 2014). Moreover, bacitracin degrades nucleic acids, in particular RNA, through a hydrolytic mechanism (Ciesiołka et al. 2014). Bacillorin and bacillomycin L should be considered as synonymous names for a single molecule.

Fusaricidins are depsipeptide antibiotics synthesized by the members of the genus *Paenibacillus*. They have a ring structure. These antibiotics have high antifungal activity against plant pathogenic fungi, such as *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus oryzae*, and *Penicillium thomii*. Fusaricidins also have good bactericidal activity against Gram-positive bacteria, such as *Staphylococcus aureus* (Li et al. 2007; Choi et al. 2008).

Tridecaptins are a class of linear cationic lipopeptides exhibiting strong activity against multidrug-resistant Gram-negative bacteria. At the same time, they show low cytotoxicity and hemolytic activity. Tridecaptins are produced by *Paenibacillus polymyxa* strain (Cochrane et al. 2015).

Most NRPS gene sequences from *Bacillus* spp. 9A, 2A and 2B strains were homologous with the sequences responsible for the synthesis of different lipopeptide biosurfactants, such as fengycin, bacillomycin, plipastatin, surfactin, and iturin. Notably, the closest relatives of the sequences of NRPS gene fragments from *Bacillus* spp. 9A and 2A strains included PKS genes responsible for the synthesis of bacillaene. The identification of PKS genes also indicated the genes responsible for the synthesis of this antibiotic. As mentioned above, a type

I PKS-NRPS hybrid gene cluster is responsible for its synthesis. Therefore, in the strains studied by two different pairs of primers, we detected the genes responsible for the synthesis of bacilaene.

The fengycin family includes fengycin and plipastatin, which are cyclic lipopeptides produced by *B. subtilis* (Bie et al. 2009). Natural fengycin is a mixture of isoforms, which differ slightly in their physicochemical properties due to variations in the chain length and branching of its hydroxy fatty acid component (Bie et al. 2009). Fengycin specifically inhibits filamentous fungi; its hemolytic activity is 40-fold less than that of surfactin (Bie et al. 2009).

Plipastatin, an antifungal antibiotic, is one of the most important non-ribosomal lipopeptides produced by *B. subtilis*. Plipastatin is involved in inhibition of phospholipase A2 and biofilm formation (Batool et al. 2011). It is produced by different strains of *Bacillus* species and shows moderate surfactant properties. It is an antifungal metabolite and inhibits filamentous fungi, but it has no effect on yeast and bacteria (Romero et al. 2007; Chen et al. 2009).

The iturin family includes compounds of iturin and bacillomycin. Both are cyclic lipopeptides produced by *B. subtilis*, and they exhibit strong antifungal properties (Peypoux et al. 1981; Zhang et al. 2013). Iturin has low toxicity in mammals and shows strong antibiotic activity, thus making it potentially a useful and effective substance for biological control to reduce the use of chemical pesticides in agriculture (Romero et al. 2007; Ongena and Jacques 2008; Kim et al. 2010; Zhang et al. 2013).

The surfactin family are structurally cyclic peptides with a multiple biological activity produced by some *B. subtilis* strains (Cosmina et al. 1993; Ongena and Jacques 2008). Surfactin is a strong surface-active compound. It can lyse erythrocytes and protoplasts of bacteria. Additionally, surfactin inhibits the thrombin-fibrinogen interaction, thus slowing the formation of fibrin. This property defines it as a possible component in the development of anticoagulants for the prevention of thromboses and diseases, such as myocardial infarction, pulmonary embolism, etc. Surfactin exhibits anticholesterol activity and decreases the level of cholesterol in the plasma and liver. It has antitumor, fungicidal, and antibiotic activity. Many useful physicochemical characteristics of this substance indicate that it can be widely used in the pharmaceutical, technical, and environmental fields.

In this study, we showed the presence of RKS and NRPS genes in the genomes of heterotrophic bacteria isolated from epilithic biofilms in Lake Baikal. The occurrence of these genes in bacteria of the genera *Bacillus* and *Paenibacillus* was higher than in other bacterial groups. Comparative analysis of the obtained amino acid sequences showed a wide variety of the genes. These sequences were related to the genes involved in biosynthesis of antibiotics (bacillaene, difficidine, erythromycin, sorangicin, and batumin), biosurfactants (fengycin, bacillomycin, plipastatin, surfactin, and iturin) and antitumor agents (epothilone, calyculin, and briostatin). *Bacillus* sp. 9A (iturin, bacillomycin, surfactin, bacillaene, and difficidine) and *Bacillus* sp. 2A (plipastatin, bacillaene, surfactin, fengycin, and difficidine) showed the highest variety of PKS and NRPS genes. Furthermore, the investigated strains exhibited multiple enzymatic and antagonistic activities, indicating that they are potential producers of bioactive metabolites. Therefore, Baikal representatives of the genera *Bacillus* and *Paenibacillus* can be of practical interest for biotechnological purposes. To confirm our assumptions, it is necessary to obtain individual compounds and determine their structure, as well as study biological activity.

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**Conflict of interest**

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

# Microbiology and Drug Resistance of Pathogens in Patients Hospitalized at the Nephrology Department in the South of Poland

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Abstract

A retrospective study was conducted among 498 patients with urinary tract infections (UTI) referred to our department from January 2013 to December 2015. This study was performed to evaluate the etiology of UTI and the antibiotic susceptibility profile of *Escherichia coli* (*E. coli*) as the main etiological factor in different age groups. Urine samples were examined using standard microbiological methods. Three hundred sixty-three samples (72.9%) were identified as *E. coli*, of which 29 (8.0%) can produce extended-spectrum β-lactamases (ESBL). *E. coli* was highly sensitive to imipenem (100.0%), gentamicin (91.0%), nitrofurantoin (89.4%), amikacin (88.2%), piperacillin/tazobactam (87.0%) and cephalosporins (79.7–89.5%). Low sensitivity was found in relation to fluoroquinolones (60.3–70.4%). *E. coli* was least sensitive to ampicillin (30.2%) and amoxicillin/clavulanic acid (49.9%). We observed a significant fall in susceptibility level to piperacillin/tazobactam (68.4% vs. 88.8%; $p = 0.017$), amikacin (61.1% vs. 90.7%; $p = 0.001$), gentamicin (70.0% vs. 93.2%; $p = 0.002$), cefalexin (41.2% vs. 83.3%; $p < 0.001$), cefotaxime (63.6% vs. 89.4%; $p = 0.002$), ceftazidime (61.9% vs. 85.6%; $p = 0.008$), cefepime (73.7% vs. 91.1%; $p = 0.025$), ciprofloxacin (54.1% vs. 72.2%; $p = 0.024$) and norfloxacin (40.5% vs. 62.5%; $p = 0.011$) among patients with catheter-associated UTI (CAUTI) compared to those with non-CAUTI. A similar susceptibility profile was observed between different age groups. In the longevity, *E. coli* showed a higher sensitivity to cephalosporins than in the young-old group. *E. coli* susceptibility to fluoroquinolones was low, which excludes them as a first-line drug in our department. Nitrofurantoin may be used as an alternative drug to carbapenems. Monitoring of susceptibility pattern is of great importance.

Key words: urinary tract infection (UTI), *Escherichia coli*, antimicrobial susceptibility testing (AST)

## Introduction

Urinary tract infections (UTI) are among most common bacterial diseases both in community and hospital settings. Due to their high rate of frequency, recurrence, complications as well as increasing antimicrobial resistance, they pose a real challenge to medical professionals. Older people are more susceptible to UTI because their immune system is weaker, and comorbidities are often present (Aplay et al. 2018). *Escherichia coli* (*E. coli*) remains the predominant isolated uropathogen in Poland accounting for 80% of all uncomplicated infections (Stefaniuk et al. 2016). However, in the presence of a urinary catheter, the spectrum of *E. coli* accounts for approximately 50% of total catheter-associated UTI (CAUTI), and other uropathogens become more prevalent (Flores-Mireles et al. 2015).

Of all hospital-associated UTI, 70–80% result from an indwelling urinary catheter, especially in older people, after surgical procedures and among patients staying in intensive care units (Temiz et al. 2012; Wójkowska-Mach et al. 2013; Piechota 2016). Catheter use is also associated with noninfectious outcomes, including mechanical trauma, mobility impairment as well as urethral strictures (Esposito et al. 2011; Hollingsworth et al. 2013). Additionally, prolonged catheterization increases the risk of biofilm formation in which uropathogens are difficult to treat with antimicrobial

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agents (Zalewska-Piatek et al. 2009). Therefore, before deciding to catheterize the bladder it is important to estimate indications individually.

An optimal empirical therapy for UTI requires understanding of local epidemiology and antimicrobial susceptibility profiles. Uropathogens have developed resistance mechanisms to commonly prescribed antibiotics what limits treatment options of effective therapies. So, we conducted a study to describe clinical characteristics of patients with UTI, including CAUTI, as well as to determine etiology and susceptibility profiles of most common urinary isolates in different age groups.

## Experimental

### Materials and Methods

A retrospective study was conducted on hospitalized adults from 1st January 2013 to 31st December 2015 in a Department of Internal Medicine and Nephrology with Dialysis Centre of Regional Hospital in southern Poland. During this period 4512 patients (2452 women and 2060 men) were hospitalized in our department in whom we diagnosed 498 cases of UTI. Among these cases, a group with CAUTI was distinguished. In the study, we made a systematic analysis of medical records of patients with diagnosed UTI.

UTI was diagnosed based on a positive result of a urine culture test with significant bacterial growth $\geq 10^5$ CFU/ml and a presence of at least one of the following symptoms: body temperature ≥ 37.5°C, dysuria, perineal pain, suprapubic pain or flank pain. Laboratory tests usually showed the elevated level of inflammatory markers and leucocyturia in urinalysis. According to the European Centre for Disease Prevention and Control (ECDC 2015), diagnostic criteria for CAUTI included the maintenance of a catheter in the bladder for at least 7 days. The patients were also categorized by age as follows: 19–74 years (young old), 75–85 years (old old) and > 85 years (longevity).

Urine for bacteriological examination was obtained from the middle stream but in catheterized patients after a catheter replacement with the hygiene and sterility principles preserved. All urine samples were inoculated in a Microbiology Department on Columbia agar with 5% sheep blood, Sabouarud agar and Chromogenic media. Inoculated agar plates were incubated aerobically at 35–37°C for 18–24 hours. Colonies were counted on the inoculated medium and multiplied by the loop volume to determine bacterial count. Bacterial identification was done by standard biochemical procedures, including Vitek 2 Compact system.

Antibiotic susceptibility testing (AST) as an *in vitro* assessment of activity of the antimicrobial drug was determined using the disc-diffusion and diffusion-gradient (E-test) methods. Zone diameters for individual antimicrobial agents were translated into susceptible, intermediate and resistant categories. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST), intermediate sensitivity strains were classified as resistant. A susceptibility level was calculated by dividing the number of sensitive strains to the antimicrobial agent by the total number of strains in relation to which the sensitivity to a particular antimicrobial agent was determined. Strains were tested against the following antibiotics: ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, amikacin, gentamicin, cefalexin, cefuroxime, cefotaxime, ceftazidime, cefepime, ciprofloxacin, norfloxacin, cotrimoxazole, imipenem and nitrofurantoin.

Because of *E. coli* predominance in the studied population, we analyzed susceptibility pattern only in reference to this uropathogen, including also division into age groups. The remaining microbes were not representative enough to be analyzed in this issue.

We accepted $p < 0.05$ as the cut-off value for the level of statistical significance. The logistic regression test and *chi*-square test were used to compare independent variables. The calculations were carried out by the SPSS software (Statistical Package for the Social Sciences, STATISTICS 20, Armonk, NY, USA).

## Results

Among 4512 hospitalized patients, we recognized 498 cases of UTI (11.0%), of which 60 were CAUTI (1.3%). The mean age was 74.8 ± 14.6 years (ranging from 19 to 101 years) without differences between CAUTI and non-CAUTI population (76.2 ± 10.4 vs. 74.6 ± 15.0 years; $p = 0.988$). Hospitalization time was significantly longer in patients with CAUTI compared to those with non-CAUTI (13.63 ± 10.47 vs. 9.58 ± 6.66 days; $p = 0.002$).

Table I presents the demographic and clinical characteristics of the studied population. Females showed much higher prevalence of the total UTI as 319 (64.1%), and in the non-CAUTI group than in the CAUTI group (67.6% vs. 38.3%; $p < 0.001$). However, males were more common in the CAUTI group (61.7% vs. 32.4%; $p < 0.001$). Patients who developed catheter-associated infections had more frequently genitourinary tumors (15.0% vs. 7.3%; $p = 0.047$) and urine stasis in kidneys (8.3% vs. 4.1%; $p = 0.001$). In addition, 10 men were after prostatectomy. No significance was found for other diseases and risk factors.

*E. coli* was the predominant isolated uropathogen in the studied population (72.9%). However, its occurence in the CAUTI group was significantly smaller com-

Table I
Characteristic of patients with UTI enrolled in the study.

| Characteristic | Total UTI n = 498 (100%) | | CAUTI n = 60 (100%) | | non-CAUTI n = 438 (100%) | | OR (95% CI) | *p*-value |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | | |
| Gender: Male | 179 | 35.9% | 37 | 61.7% | 142 | 32.4% | 3.35 (1.92–5.86) | < 0.001 |
| Female | 319 | 64.1% | 23 | 38.3% | 296 | 67.6% | 0.30 (0.17–0.52) | < 0.001 |
| Hypertension | 263 | 52.8% | 37 | 61.7% | 226 | 51.6% | 1.51 (0.87–2.62) | 0.145 |
| Heart failure | 174 | 34.9% | 23 | 38.3% | 141 | 32.2% | 1.31 (0.75–2.29) | 0.344 |
| Diabetes mellitus | 190 | 38.2% | 18 | 30.0% | 172 | 39.3% | 0.72 (0.40–1.28) | 0.258 |
| Urolithiasis | 22 | 4.4% | 1 | 1.7% | 21 | 4.8% | 0.34 (0.04–2.55) | 0.292 |
| Bronchopneumonia | 9 | 1.8% | 1 | 1.7% | 8 | 1.8% | 0.91 (0.11–7.41) | 0.931 |
| Malignancy (total) | 80 | 16.1% | 11 | 18.3% | 69 | 15.8% | 1.34 (0.68–2.65) | 0.405 |
| Genitourinary malignancy | 41 | 8.2% | 9 | 15.0% | 32 | 7.3% | 2.24 (1.01–4.96) | 0.047 |
| Prostatic hyperplasia | 57 | 11.4% | 10 | 16.7% | 47 | 10.7% | 1.66 (0.79–3.50) | 0.180 |
| Urine stasis in kidneys | 23 | 4.6% | 5 | 8.3% | 18 | 4.1% | 4.34 (1.76–10.73) | 0.001 |
| Percutaneous nephrostomy | 8 | 1.6% | 1 | 1.7% | 7 | 1.6 % | 1.04 (0.13–8.63) | 0.968 |
| Hemodialysis | 20 | 4.0% | 2 | 3.3% | 18 | 4.1% | 0.80 (0.18–3.56) | 0.774 |
| Immunotherapy | 26 | 5.2% | 4 | 6.7% | 22 | 5.0% | 1.35 (0.45–4.06) | 0.593 |

Data are expressed as number and percentage.
One patient may have several co-existing diseases

Table II
Microbial uropathogens isolated from the urine samples.

| Pathogen | Total UTI | | CAUTI | | non-CAUTI | | OR (95% CI) | *p*-value |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | | |
| *E. coli* | 363 | 72.9% | 37 | 61.7% | 326 | 74.4% | 0.55 (0.31–0.97) | 0.039 |
| *Proteus* sp. | 30 | 6.0% | 6 | 10.0% | 24 | 5.5% | 1.92 (0.75–4.90) | 0.174 |
| *Staphylococcus* sp. | 12 | 2.4% | 5 | 8.3% | 7 | 1.6% | 5.60 (1.72–18.24) | 0.004 |
| *Enterobacter* sp. | 20 | 4.0% | 4 | 6.7% | 16 | 3.7% | 1.88 (0.61–5.84) | 0.272 |
| *Klebsiella* sp. | 40 | 8.0% | 2 | 3.3% | 38 | 8.7% | 0.36 (0.09–1.54) | 0.170 |
| *Enterococcus* sp. | 11 | 2.2% | 2 | 3.3% | 9 | 2.0% | 1.64 (0.35–7.80) | 0.532 |
| Other | 22 | 4.4% | 4 | 6.7% | 18 | 4.1% | 1.67 (0.54–5.10) | 0.371 |
| Total | 498 | 100.0% | 60 | 100.0% | 438 | 100.0% | – | – |

Other pathogens:
*Pseudonomas* sp. (n = 6), *Morganella morganii* (n = 4), *Acinetobacter baumanii* (n = 3), *Serratia* sp. (n = 3), *Citrobacter* sp. (n = 2), *Raoultella planticola* (n = 1), *Salmonella* (n = 1), *Stenotrophomonas maltophilia* (n = 1), *Streptococcus agalactiae* (n = 1)
ESBL-producing pathogens responsible for CAUTI: *E. coli* (n = 6), *Klebsiella* sp. (n = 2), *Enterobacter* sp. (n = 1)
ESBL-producing pathogens responsible for non-CAUTI: *E. coli* (n = 23), *Klebsiella* sp. (n = 12), *Enterobacter* sp. (n = 11), *Proteus* sp. (n = 4)

pared to non-CAUTI group (61.7% vs. 74.4%; $p = 0.039$) (Table II). The occurrence of other microbes in the CAUTI and non-CAUTI groups were much lower: *Proteus* sp. (10.0% vs. 5.5%; $p = 0.174$), *Staphylococcus* sp. (8.3% vs. 1.6%; $p = 0.004$), *Enterobacter* sp. (6.7% vs. 3.7%; $p = 0.272$), *Klebsiella* sp. (3.3% vs. 8.7%; $p = 0.170$) and *Enterococcus* sp. (3.3% vs. 2.0%; $p = 0.532$).

Production of extended-spectrum β-lactamases (ESBL) was found in 11.8% of total strains with the prevalence as follows: in the CAUTI (9/60; 15.0%) group and in the non-CAUTI (50/438; 11.4%) group. *Enterobacter* sp. were most frequently associated with ESBL production as 60.0% of all *Enterobacter* sp. isolates was positive, followed by *Klebsiella* sp. (35.0%).

Table III presents susceptibility profile of *E. coli* in the studied population to various antimicrobial agents. *E. coli* showed the highest prevalence of susceptibility to imipenem (100.0%), followed by gentamicin (91.0%), nitrofurantoin (89.4%), amikacin (88.2%), piperacillin/tazobactam (87.0%) and cephalosporins (79.7–89.5%). The prevalence of susceptibility was least to ampicillin (30.2%) and amoxicillin/clavulanic acid (49.9%). We

Table III
Susceptibility profile of *E. coli* isolates studied to various antimicrobial agents.

| Antibiotics | Total UTI (n = 363) | | CAUTI (n = 37) | | non-CAUTI (n = 326) | | OR (95% CI) | *p*-value |
|---|---|---|---|---|---|---|---|---|
| | n' | % susc. | n' | % susc. | n' | % susc. | | |
| Beta-lactam antibacterials, penicylinns | | | | | | | | |
| Ampicillin | 354 | 30.2% | 36 | 19.4% | 318 | 31.4% | 0.53 (0.22–1.24) | 0.143 |
| AM/CL | 361 | 49.9% | 37 | 43.2% | 324 | 50.6% | 0.74 (0.37–1.48) | 0.397 |
| PIP/TZ | 207 | 87.0% | 19 | 68.4% | 188 | 88.8% | 0.27 (0.09–0.79) | 0.017 |
| Aminoglycosides | | | | | | | | |
| Amikacin | 211 | 88.2% | 18 | 61.1% | 193 | 90.7% | 0.16 (0.06–0.47) | 0.001 |
| Gentamicin | 211 | 91.0% | 20 | 70.0% | 191 | 93.2% | 0.17 (0.06–0.52) | 0.002 |
| Cephalosporins | | | | | | | | |
| Cefalexin | 197 | 79.7% | 17 | 41.2% | 180 | 83.3% | 0.14 (0.05–0.40) | < 0.001 |
| Cefuroxime | 358 | 85.8% | 36 | 75.0% | 322 | 87.0% | 0.45 (0.20–1.02) | 0.057 |
| Cefotaxime | 238 | 87.0% | 22 | 63.6% | 216 | 89.4% | 0.21 (0.08–0.55) | 0.002 |
| Ceftazidime | 237 | 83.5% | 21 | 61.9% | 216 | 85.6% | 0.27 (0.10–0.71) | 0.008 |
| Cefepime | 210 | 89.5% | 19 | 73.7% | 191 | 91.1% | 0.27 (0.09–0.85) | 0.025 |
| Antipseudomonal fluoroquinolones | | | | | | | | |
| Ciprofloxacin | 361 | 70.4% | 37 | 54.1% | 324 | 72.2% | 0.45 (0.23–0.90) | 0.024 |
| Norfloxacin | 360 | 60.3% | 37 | 40.5% | 323 | 62.5% | 0.41 (0.20–0.82) | 0.011 |
| Folate pathway inhibitors | | | | | | | | |
| Cotrimoxazole | 358 | 72.3% | 36 | 72.2% | 322 | 72.4% | 0.99 (0.46–2.14) | 0.986 |
| Antipseudomonal carbapenems | | | | | | | | |
| Imipenem | 179 | 100.0% | 18 | 100.0% | 161 | 100.0% | – | – |
| Nitrofuran derivatives | | | | | | | | |
| Nitrofurantoin | 357 | 89.4% | 37 | 83.8% | 320 | 90.0% | 0.57 (0.22–1.48) | 0.251 |

Abbrevations: Amoxicillin/clavulanic acid = AM/CL, Piperacillin/Tazobactam = PIP/TZ
n' – number of all determinations for a given antibiotic; % susc. – % susceptibility

also observed a fall in sensitivity level of *E. coli* to antibiotics among CAUTI patients compared to non-CAUTI individuals, with the significant differences in relation to piperacillin/tazobactam (68.4% vs. 88.8%; $p = 0.017$), amikacin (61.1% vs. 90.7%; $p = 0.001$), gentamicin (70.0% vs. 93.2%; $p = 0.002$), cefalexin (41.2% vs. 83.3%; $p < 0.001$), cefotaxime (63.6% vs. 89.4%; $p = 0.002$), ceftazidime (61.9% vs. 85.6%; $p = 0.008$), cefepime (73.7% vs. 91.1%; $p = 0.025$), ciprofloxacin (54.1% vs. 72.2%; $p = 0.024$) and norfloxacin (40.5% vs. 62.5%; $p = 0.011$).

An antimicrobial susceptibility profile of *E. coli* depending on age groups was summarized in Table IV. The youngest group constituted the reference for other groups. *E. coli* showed a similar susceptibility level to most antimicrobial agents between different age groups. In the longevity, *E. coli* demonstrated higher sensitivity to cephalosporins than in the young-old group, with significant differences relating to cefalexin (93.9% vs. 74.3%; $p = 0.011$), cefotaxime (96.7% vs. 81.9%; $p = 0.017$) and cefepime (98.2% vs. 84.9%; $p = 0.033$).

Furthermore, we also carried out an analysis concerning *E. coli* sensitivity to antimicrobial agents in patients with CAUTI and non-CAUTI, according to age groups. No significant variations in drug susceptibility were noted among patients with catheter-associated infections between age groups. The only exception concerned the longevity group with non-CAUTI, in whom *E. coli* showed significantly higher prevalence of susceptibility than in the young old group, relating to cefalexin ($p = 0.019$) and cefotaxime ($p = 0.034$).

## Discussion

Urinary tract infection is emerging as an important community-acquired and nosocomial bacterial infection what was also confirmed by our 3-year analysis. It occurs on average in 1 per 10 of hospitalized patients and that was also presented in our previous study (Michno et al. 2016).

In our study, UTI mainly concerned older people who constituted the majority, as evidenced by average age of the studied population. The proportion of older people is rising constantly from 11% in 2012 and it is

Table IV
Susceptibility profile of *E. coli* (n = 363) isolated from patients of different age groups.

| Antibiotics | 19–74 years (n = 133) | | 75–85 years (n = 134) | | > 85 years (n = 96) | | 75–85 years vs. 19–74 years | | > 85 years vs. 19–74 years | |
|---|---|---|---|---|---|---|---|---|---|---|
| | n' | % susc. | n' | % susc. | n' | % susc. | OR (95% CI) | *p*-value | OR (95% CI) | *p*-value |
| Ampicillin | 132 | 31.1% | 130 | 26.9% | 92 | 33.7% | 0.82 (0.48–1.40) | 0.461 | 1.13 (0.64–1.99) | 0.678 |
| AM/CL | 133 | 49.6% | 133 | 51.1% | 95 | 48.4% | 1.06 (0.66–1.72) | 0.806 | 0.95 (0.56–1.61) | 0.858 |
| PIP/TZ | 73 | 86.3% | 80 | 85.0% | 54 | 90.7% | 0.90 (0.36–2.23) | 0.819 | 1.56 (0.50–4.85) | 0.446 |
| Amikacin | 74 | 90.5% | 83 | 81.9% | 54 | 94.4% | 0.47 (0.18–1.24) | 0.127 | 1.78 (0.44–7.21) | 0.422 |
| Gentamicin | 72 | 88.9% | 85 | 89.4% | 54 | 96.3% | 1.06 (0.38–2.89) | 0.916 | 3.25 (0.66–15.97) | 0.147 |
| Cefalexin | 70 | 74.3% | 78 | 75.6% | 49 | 93.9% | 1.07 (0.51–2.26) | 0.849 | 5.31 (1.47–19.19) | 0.011 |
| Cefuroxime | 132 | 85.6% | 133 | 82.0% | 93 | 91.4% | 0.76 (0.40–1.47) | 0.421 | 1.79 (0.75–4.28) | 0.193 |
| Cefotaxime | 83 | 81.9% | 95 | 85.3% | 60 | 96.7% | 1.28 (0.58–2.83) | 0.548 | 6.40 (1.40–29.14) | 0.017 |
| Ceftazidime | 83 | 80.7% | 94 | 81.9% | 60 | 90.0% | 1.08 (0.51–2.31) | 0.839 | 2.15 (0.79–5.87) | 0.136 |
| Cefepime | 73 | 84.9% | 82 | 87.8% | 55 | 98.2% | 1.28 (0.51–3.21) | 0.602 | 9.58 (1.20–76.64) | 0.033 |
| Ciprofloxacin | 132 | 72.0% | 134 | 67.9% | 95 | 71.6% | 0.82 (0.49–1.39) | 0.471 | 0.98 (0.55–1.76) | 0.949 |
| Norfloxacin | 133 | 62.4% | 132 | 59.1% | 95 | 58.9% | 0.87 (0.53–1.43) | 0.581 | 0.86 (0.50–1.48) | 0.598 |
| Cotrimoxazole | 130 | 74.6% | 133 | 67.7% | 95 | 75.8% | 0.71 (0.42–1.22) | 0.215 | 1.06 (0.58–1.97) | 0.841 |
| Imipenem | 57 | 100.0% | 74 | 100.0% | 48 | 100.0% | – | – | – | – |
| Nitrofurantoin | 132 | 91.7% | 131 | 87.8% | 94 | 88.3% | 0.65 (0.29–1.47) | 0.303 | 0.69 (0.28–1.66) | 0.402 |

Abbrevations: Amoxicillin/clavulanic acid = AM/CL, Piperacillin/Tazobactam = PIP/TZ
n' – number of all determinations for a given antibiotic; % susc. – % susceptibility

supposed to reach 22% by 2050 (UN 2012). Thus, challenges associated with infections in older population require specific assessment.

Urinary tract catheterization is one of the most common reason of bacteriuria caused by tendency of bacteria to adhere to artificial materials. It is believed that CAUTI occurs at a rate of 3–7% per day of catheterization and the incidences approach 100% within 30 days (Lo et al. 2014). Among patients hospitalized in our department, CAUTI were diagnosed in 1.3% cases that was a slightly higher than in the study carried out in Australia – 0.9% (Gardner et al. 2014). In another study, CAUTI were reported in 2.2% of hospitalized patients of the urological and orthopedic department (Giles et al. 2015).

In the current study, CAUTI was significantly more frequent in men that was similar to the Korean report (Kim et al. 2017). The authors, like in our study, analyzed the characteristics of CAUTI and non-CAUTI patients. They observed that CAUTI occurred significantly more often in patients with hypertension, those who used ventilators and after operations. In our analysis, CAUTI was more frequent in patients with genitourinary malignancy and urine stasis in kidneys. Patients with cancer have a greater tendency to acquire infections than general population due to cellular and humoral immune dysfunction, as well as complications of cancer therapy, including neutropenia or disruption of natural physical barriers (Thirumala et al. 2010). Urinary tract obstruction provides an opportunity for bacteria to adhere to urothelium and infect patients. In such case, effective antibiotic therapy as well as an appropriate urological intervention is necessary to prevent recurrent UTI and septic complications.

The predominant uropathogen responsible for CAUTI in our study was *E. coli*, followed by *Proteus* sp. and *Staphylococcus* sp. In patients with non-CAUTI, apart from *E. coli*, we also frequently observed infections caused by *Klebsiella* sp. and *Proteus* sp. A lot of authors indicate a very similar bacterial flora (Krygiel et al. 2012; Kalal and Nagaraj 2016; Wang et al. 2016).

*E. coli* was the main etiological factor in both groups, however, its predominance was significantly smaller in catheter-associated infections. This is because CAUTI belong to complicated ones with higher rate of hospital-acquired infections, in which prevalence of the *E. coli* is not as high as in uncomplicated UTI (Holecki et al. 2015). In such a situation, other pathogens which are less commonly associated with UTI become more prevalent. Our study showed a significant increase of *Staphylococcus* sp. isolates in patients with CAUTI ($p = 0.004$). Recent studies highlighted that *Staphylococcus aureus* is a common problem associated with urinary catheterization, leading more often to bacteremia than other uropathogens (Muder et al. 2006; Baraboutis et al. 2010).

*Proteus* sp. were the second most prevalent cause of CAUTI and occurred almost twice more often than in the non-CAUTI in this study. According to some

reports, *Proteus mirabilis* is also supposed to be a frequent reason of CAUTI, especially in males, with a tendency to biofilm formation accounting for 17.9% of *Enterobacteraceae* family (Jacobsen et al. 2008; Moryl et al. 2013).

In the current study, *Klebsiella* sp. were the second common cause of non-CAUTI which is compatible with most publications (Farajnia et al. 2009; Abou-Dobara et al. 2010). However, another study reported that *Pseudomonas* sp. were the second frequent isolated microorganisms responsible for 8.7% of total UTI (Abejew et al. 2014).

Antimicrobial resistance is now accepted as a major problem in public health and patient care. It is mainly associated with an abuse of antimicrobial agents and makes it difficult to choose a proper empirical treatment by medical practitioners, especially in cases of multidrug resistant strains (Pobiega et al. 2015). Uropathogens developed resistance mechanisms of which the most common is ESBL. Organisms producing ESBL are clinically relevant and remain an important cause of failure of cephalosporins (Bradford 2001). In this study, *Enterobacter* sp. and *Klebsiella* sp. were the most frequently ones associated with ESBL production of all these species, followed by *Proteus* sp. and *E. coli*. Similar results were also reported by other authors from Poland (Sacha et al. 2007). *E. coli* as the predominant uropathogen was responsible for ESBL production in 8.0% of total *E. coli* isolates being positive, similarly to the previous data from southern Poland (Pobiega et al. 2013). For example, in France approximately 4.0% of *E. coli* isolates are ESBL producers, while in Mexico even 31.3% of *E. coli* isolates produce ESBL mechanism (Galindo-Méndez 2018; Zucconi et al. 2018).

Susceptibility of *E. coli* in the studied population was highest to imipenem and gentamicin (> 90.0%), and lowest to ampicillin as well as amoxicillin/clavulanic acid (< 50.0%). This is similar to the reports from other countries with the exception of gentamicin which was a less effective drug (Daoud et al. 2015; Li et al. 2017). It may be since in our country gentamicin is rarely used as the first empiric choice for UTI treatment. Although, imipenem was the most effective drug, it should not be administrated as an empirical drug unless infection is life threatening, as carbapenems are considered drugs of last resort.

It is important to point out that the minimum susceptibility rate to support empirical treatment of UTI is 80% of all the strains of a specific uropathogen in a given region. According to these guidelines, the European Association of Urology (EAU) recommend cotrimoxazole as a first-line drug for empirical therapy in UTI (Grabe et al. 2009). In our study, 74.6% of *E. coli* isolates were susceptible to cotrimoxazole. Despite the fact that these values may vary among reports, the resistance rate of *E. coli* to cotrimoxazole in Europe tends to be above 20%, having also been reported higher than 30% (Guneysel et al. 2009; Schito et al. 2009). *E. coli* susceptibility to fluoroquinolones in the current study ranged from 60.3% (norfloxacin) to 70.4% (ciprofloxacin) which was similar to the rates observed in Brazil and Korea (Reis et al. 2016; Park et al. 2017). Another study reported that *E. coli* was least sensitive to cephalosporins (7.0–34.0%) and fluoroquinolones (26.0–28.0%); however, a study was conducted on a population from low socioeconomic strata (Kidwai et al. 2017). A good empirical choice seems to be nitrofurantoin but a study carried out in Poland showed a high proportion of resistance to nitrofurantoin among *E. coli* isolates (Stefaniuk et al. 2016). Amikacin, as an infusion solution, may by administrated only in hospital settings. Cephalosporins also remain a good option for empirical treatment of UTI caused by *E. coli* because of relatively high sensitivity to this group of drugs and minor side effects. On the other hand, cephalosporins are commonly prescribed by medical professionals which causes a growing resistance to them among bacteria. Therefore, clinical practitioners as well as laboratory personnel should implement a program to detect and report resistant strains such as ESBL producing bacteria to control and limit the therapeutic failures.

Patients with CAUTI are a specific group in which treatment of the infection may by difficult due to a high resistance rate of uropathogens. In such a case, it is necessary to administer an adequate initial antimicrobial therapy according to local antimicrobial susceptibility situation (Saurel et al. 2006). Inadequate empirical antimicrobial therapy extends treatment contributing to various complications and transformation into a chronic illness. Our analysis confirmed that *E. coli* isolates were significantly less sensitive to most antibiotics in the CAUTI group compared to non-CAUTI group. Nitrofurantoin (83.8%) besides imipenem (100.0%), seemed to be an optimal choice in cases of catheter-associated infections caused by *E. coli*, contrary to ampicillin (19.4%). This result is compatible with another report (Albu et al. 2018). However, other authors found amikacin (100.0%) and cefepime (100.0%) to be superior to nitrofurantoin (92.8%) in susceptibility rate what is incompatible with our analysis (Piljic et al. 2013).

Regardless of the age groups among the population analyzed in this study, *E. coli* showed sensitivity to most antibiotics, including: imipenem, nitrofurantoin, amikacin, gentamicin, piperacillin/tazobactam as well as cephalosporins. Moreover, in reference to our results, it may be helpful for medical practitioners to choose cefalexin, cefotaxime or cefepime in case of UTI caused by *E. coli* in the longevity group, because of their significantly higher susceptibility rate compared to young-old

as well as old-old groups. Fluoroquinolones in our report turned out to have limited efficacy among patients with UTI, regardless of the age group. Thus, choosing them as first-line drugs in empiric therapy raises doubts and should be careful in admitted patients.

The results of this study can be used to construct a hospital formulary and internal procedure for antibiotics usage in case of UTI in a specific department. Our results can also be helpful for comparison between departments and hospitals interested in this issue.

There are some limitations to this study. First, we retrospectively collected data through electronic medical records. It was difficult to obtain all characteristics for analyzing risk factors such as previous antibiotics use and history of recurrent UTI due to unrecorded information. Second, this study was conducted only in a single center. Therefore, it is difficult to reflect the overall characteristics of our region. The current report suggests the need for further large-scale monitoring of epidemiology and susceptibility profiles of most common uropathogens causing UTI, including CAUTI, to improve the effectiveness of empirical treatment.

## Conclusions

The presented study showed considerable bacterial resistance to common empirically used antibiotics in case of CAUTI. *E. coli* susceptibility to fluoroquinolones was relatively low, which indicates the necessity of exclusion this group of drugs as first-line for the empirical treatment of UTI in our department. Nitrofurantoin as an alternative to carbapenems, can be used in empiric therapy of UTI, including CAUTI, regardless of the age group. Monitoring of susceptibility pattern is of great importance.

**Conflict of interest**

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ORIGINAL PAPER

# A Special Risk Group for Hepatitis E Infection: The First Record of North Cyprus

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Abstract

Hepatitis E virus (HEV) is transmitted by a fecal oral route from animals to humans following exposure to the body fluids of infected animals. We investigated the seroprevalence of anti-hepatitis E (anti-HEV) antibodies by monitoring IgG and IgM virus antibodies amongst employees in the animal industry in North Cyprus through a cross-sectional study. Samples were taken from individuals without occupational exposure to animals and from those who worked with animals (doing animal husbandry, veterinary work or butchery). Enzyme-linked immunoassays were used to detect anti-HEV IgG and IgM in the blood samples. The prevalence of anti-HEV IgG antibodies was 3.0% (12/400), while the prevalence of anti-HEV IgM antibodies was 0.25% (1/400). The prevalence of anti-HEV IgG amongst the samples received from females was approximately 2.5-fold higher than samples received from males (2.4%). Anti-HEV IgG was detected amongst 7% of animal husbandry workers and amongst 2% of veterinarians and butchers. The current findings represent the first records of HEV surveillance in Cyprus. We investigated the seroprevalence of anti-HEV by monitoring IgG and IgM virus antibodies amongst employees.

Key words: Cyprus, hepatitis E, seroprevalence, zoonoses, animals, epidemiology

## Introduction

Hepatitis E (HEV) infection involves a non-enveloped virus belonging to the genus of *Orthohepevirus*. It is a zoonotic pathogen which has spread through a number of developing countries (Wu et al. 2016). The virus has been designated with the letter E for its transmission via the enteric route (Lapa et al. 2015). Currently, it is estimated that 20 million individuals worldwide are infected with HEV, where it is the cause of 57 000 deaths each year (Lapinski et al. 2016). It is also responsible for 3.3 million new symptomatic infections with fatal outcomes in 56 600 individuals (Mauceri 2018). The World Health Organization (WHO) has reported that the low endemic regions of HEV epidemiology are found in the USA and Europe (the UK, France, the Netherlands, Austria, Spain, Greece and Germany) as well as developed Asian-Pacific countries (Japan, Taiwan, Korea, Hong Kong, Australia and New Zealand) (WHO 2010). High endemic regions are found in Central America, Africa and South and Central Asia (Melgaco 2018). The prevalence of anti-HEV IgG in Africa is between 4.6% and 10.7% and is between 34.8% and 94% in Asia (Melgaco 2018).

The presence of anti-HEV IgM was conclusive at the onset of the infection and increased by the beginning of the fourth week; however, no further increase was detected after three months. Anti-HEV IgG antibodies increase to their peak level by the end of the fourth week and can continue for years (El-Tras et al. 2013). HEV is generally transmitted by the fecal-oral route, following transfusion with infected blood products, through vertical transmission and also through contact with infected animals (Hesamizadeh et al. 2016). Individuals considered to be at high risk of contracting the virus include employees of slaughterhouses, people working on pig farms and veterinarians (Holt et al. 2016).

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The aim of the study was to determine the health risks posed to employees working with animals in North Cyprus by monitoring the prevalence of HEV.

## Experimental

### Materials and Methods

**Setting.** The present study was a cross-sectional survey of animal workers from five regions in North Cyprus. The distribution of participants across the different districts in North Cyprus was as follows: Nicosia (28.8%), Kyrenia (18%), Omorphou (11%), Famagusta (21.7%) and Trikomo (20.5%) according to animal workers numbers supplied by Veterinary Office of North Cyprus. Since HEV prevalence may be as low as 1%, taking $\alpha = 0.05$ and $\beta = 0.20$ one could calculate the minimum number of people to be investigated as 400. Therefore, four hundred persons were selected who did not have any clinical findings according to general health state. Individuals participating in the study were categorized into four groups based on the level of interaction each participant had with animals in their work life. Group 1 included participants without occupational exposure to animals, while Group 2 consisted of participants who worked with animals (animal husbandry). Group 3 included veterinarians, and Group 4 was composed of butchers. All participants were asked by attained healthy worker to complete epidemiological questionnaire that included basic demographic and epidemiological data such as name, age, sex, how much years to spend as animals' workers.

**Laboratory Testing.** A total of 400 blood samples were collected. Serum samples were separated by centrifugation at 1000 × g for 10 min and were stored at –20°C until required for antibody detection. All serum samples were tested using a commercially available enzyme-linked immunoassay (ELISA) in a kit (Dia Pro, Italy), in accordance with the manufacturer's instructions. The positive and negative controls were supplied in the kit and were included in each antibody screening test. Anti-HEV IgG and anti-HEV IgM ELISA tests were performed separately. The results from all the samples were calculated as a ratio of the individual absorbance to the cut-off value (S/CO). The samples with a ratio < 1.0 were considered as negative, 1.0–1.2 as equivocal and > 1.2 as positive. The diagnostic sensitivity of > 98% was found according to manufacturer's instruction.

**Ethics Committee Approval.** Ethical approval was granted by the Institutional Research Ethics Committee of the Near East University (No. 179).

**Statistical Methods.** The software program SPSS version 3.0 (SPSS Inc. Chicago, IL, USA) was used for the statistical analyses. Student *t*-tests were used for mean differences. Data were expressed as a mean value (standard deviation), as a minimum-maximum and as a percentage, where appropriate. A *p*-value of less than 0.05 was considered statistically significant.

## Results

The mean age of participants was 39.4, with the youngest being 13 and the eldest being 84 years of age (Table I). Three percent of the samples examined were positive for anti-HEV IgG (12/400), while 0.25% of samples were tested positively for anti-HEV IgM antibodies (1/400) as shown in Table II. The 400 participants consisted of 334 males and 66 females. The prevalence of anti-HEV IgG antibodies was 6.1% (n = 4) amongst female participants and 2.4% (n = 8) amongst male participants (Table II); however, these differences were not statistically significant ($p = 0.115$). Similarly, the anti-HEV IgM seropositivity was not significantly associated with gender ($p = 0.836$). Anti-HEV IgG seropositivity was significantly associated with years spent working with animals ($p = 0.001$). Ten cases of anti-HEV IgG were found for people who spent more

Table I
The socio-demographic characteristics of groups (n = 400).

| Age in years | N (%) |
|---|---|
| < 30 | 108 (27.0) |
| 30–40 | 109 (27.2) |
| 41–50 | 80 (20.0) |
| 51–60 | 87 (21.8) |
| > 61 | 16 (4.0) |

Table II
Prevalence of the Anti-HEV IgG and IgM seropositivity among gender and time spent in contact with animals.

| | Anti-HEV IgG (+) Positive | | | Anti-HEV IgM (+) Positive | | |
|---|---|---|---|---|---|---|
| Female vs. male Female | Male | *p*-value | Female | Male | *p*-value | |
| 4 | 8 | 0.115 | 0 | 1 | 0.836 | |
| Time spent in contact with animals | < 20 years | > 20 years | *p*-value | < 20 years | > 20 years | *p*-value |
| 2 | 10 | 0.001 | 1 | 0 | 0.644 | |

Table III
Prevalence of the Anti-HEV IgG and IgM seropositivity in different locations.

| Location | IgG-positive 12 (3%) | IgM-positive 1 (0.25%) |
|---|---|---|
| Nicosia | 1 (0.01%) | 0 |
| Kyrenia | 2 (2.7%) | 0 |
| Morphou | 2 (4.5%) | 0 |
| Famagusta | 6 (6.9%) | 1 (1.5%) |
| Trikomo | 1 (1.2%) | 0 |

than 20 years in contact with animals (Table II). Concerning the geographical localization, of the 12 samples which tested positive for anti-HEV IgG antibodies, 6.9% (n = 6) were in Famagusta, 2.7% (n = 2) in Kyrenia, 0.01% (n = 1) in Nicosia, 4.5% (n = 2) in Morphou and 1.2% (n = 1) in Trikomo (Table III). Distribution of anti-HEV IgG and IgM were not significantly different according to locations ($p < 0.05$; $p = 0.101$, $p = 0.462$, respectively) (Table III).

## Discussion

Cyprus is in the Mediterranean Sea. Until the current study, there has been no surveillance of the seroprevalence and epidemic of HEV infections in Cyprus. The seroprevalence has been changing, and there is a link to geographical locations between 0.0% to 0.9% anti-HEV IgM and 1.1% to 14.2% for anti-HEV IgG depending of the region of Iran (Taherkhani and Farshadpour 2016). Outbreaks of HEV, as such, have not occurred and the prevalence of sporadic cases is lower than 25% (Mohebbi et al. 2012).

In Europe, HEV seroprevalence is predicted to be from 7.5% to 31.9%, with an average of 19.16%. Nonetheless, the real seroprevalence could change due to differences in test sensitivity and the number of asymptomatic courses of the HEV disease (Mauceri 2018). The prevalence rates of anti-HEV in Europe, especially in France (17%) and Germany (35%), could again be based on the pork consumption (Melgaco 2018). There is no published report to allow a comparison with HEV seroprevalence in the Cyprus population.

Prevalence of HEV viremia in blood donors ranges between 1/762 in the Netherlands and 1/9500 people in the United States. Especially immunosuppressed recipients are under the risk of the HEV infection by contaminated blood products (Niederhauser 2018). In Turkey, the total HEV seroprevalence rate was found to be equal to 4.4% (Aydın et al. 2016). In our control group which included blood donors, anti-HEV IgG seropositivity is 1%. This result indicated that anti-HEV seroprevalence was low in our general population. According to the previous studies, the exposure to and more specifically devouring of rare meat products are the most important risk factor for HEV transmission (Melgaco 2018). In North Cyprus, the consumption of rare or raw meat is really low which may be a reason that the seroprevalence is lower than in the other countries.

El-Tras et al. (2013) reported 26.8% seropositivity in males, although the female seropositivity was 50.8% in Egypt, and there were significant differences ($p = 0.05$). Altındiş et al. (2000) indicated that female seropositivity was 6.7% and male positivity was 3.7% in Turkey. In the current study, anti-HEV IgG antibody concentrations among females were 2.5-fold higher than those in males and this difference was not statistically significant. Our prediction found a higher anti-HEV IgG seroprevalence in female because females could be more involved in activities of supplying animals so that they were more often exposed to animals. Interestingly, anti-HEV IgG seroprevalence was high in male in our study. The majority of the anti-HEV IgG seropositive farmers live in Famagusta. Famagusta has a high proportion of people whose have own farmers. An association to transmission may exists probably through animal wastes and other tissues. Our study has some limitations since statistical studies were difficult due to low overall seroprevalence for HEV in Cyprus.

The prevalence of HEV ranged from 0 to 12.4% in Turkey. The high seroprevalence was detected in agriculture workers (35%). Leblebicioğlu and Ozaras (2018) suggested that fecal route is not a main way of transmission to the HEV in Turkey. Also, low socio-economical status could be related with the seroprevalence of the HEV. The seroprevalence rates were higher for peoples which live in camps or work in agriculture and animal husbandry (Leblebicioğlu and Ozaras 2018). There have been a few studies reported the seroprevalence of anti-HEV-IgG in agricultural workers as being in the range from 4.4% to 34.8%, a 11.5-fold higher than the control groups in Turkey (Ceylan et al. 2003). Eker et al. (2009) reported that 28.5% of seropositive cases were associated with animals such as goats, sheep and cattle. Leblebicioğlu and Ozaras (2018) reported that Turkey is among the countries where HEV is endemic, but there were some limitations to their study since they did not cover the entire population. Aydın et al. (2016) reported that seroprevalence of HEV in animal workers were 35.9% and most frequent in animal husbandry (Aydın et al. 2016). Our results demonstrated a higher anti-HEV IgG seroprevalence among those who spent over 20 years working with animals as compared to less than 20 years. Thus, the time of contact with animals is positively correlated with the risk of infection.

Studies which are designed for the surveillance of HEV antibodies in both animals and humans will

contribute to a better understanding of the seroprevalence of HEV. Close monitoring by the government of precautions when working with animals, high socioeconomical status and high educational level may be important to employees in order to decrease the prevalence of the diseases. Currently, there is no data recorded within the Health Ministry of Cyprus regarding HEV infections; thus, the current findings represent the first record of HEV surveillance in Cyprus.

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**Conflict of interest**

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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SHORT COMMUNICATION

# Detection of *Coxiella burnetii* and *Francisella tularensis* in Tissues of Wild-living Animals and in Ticks of North-west Poland

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Abstract

This work presents results of the research on the occurrence of *Coxiella burnetii* and *Francisella tularensis* in the tissues of wild-living animals and ticks collected from Drawsko County, West Pomeranian Voivodeship. The real-time PCR testing for the pathogens comprised 928 samples of animal internal organs and 1551 ticks. The presence of *C. burnetii* was detected in 3% of wild-living animals and in 0.45–3.45% (dependent on collection areas) of ticks. The genetic sequences of *F. tularensis* were present in 0.49 % of ticks (only in one location – Drawa) and were not detected in animal tissues. The results indicate respectively low proportion of animals and ticks infected with *C. burnetii* and *F. tularensis*.

Key words: *Coxiella burnetii*, *Francisella tularensis*, reservoirs, real-time PCR

Wild-living animals play a significant role in epidemiology of zoonoses; they constitute the main source of pathogens dangerous for humans and domestic animals. Number of zoonotic agents carried by wild-living animals increases and still the threat they pose is not well known, particularly for humans with direct contact to animals. The scope of danger may vary and it depends not only on the source of infection but also on transmission routes (Artois 2003; Jones et al. 2008). The presence of vectors in the environment (e.g. ticks) is correlated with the existence of ecological niches inhabited by the hosts and tick-specific environmental conditions (temperature and humidity levels) (Daszak et al. 2000). Ticks are among the most common zoonosis vectors; tick-borne diseases (TBDs) are a significant group of diseases impacting public health.

Some of the zoonosis-related threats are the diseases caused by *C. burnetii* and *F. tularensis*. *C. burnetii* can be isolated from both domestic and wild-living animals such as bears, bisons, red deers, roe deers, boars, rabbits, shrews and marsupials. The infections occur through direct contact with infected animals, by aerosol inhalation, ingestion, direct contact with wounded skin and as a result of tick bites (Norlander 2000; Bossi et al. 2004; Woldehiwet 2004). Arthropods are a significant vector of pathogen transmission; however, multiple other transmission routes exist. Infection can occur after bite of a feeding arthropod, mechanically (e.g. flies), by contact with wounded skin or by inhaling the faeces of parasites (ticks) (Marrie 1990; Anusz 1995; Mediannikow et al. 2010). *F. tularensis* is found to be important threat in forests and on farmlands; while its main reservoir are wild-living rodents. Arthropods (ticks, mites, mosquitoes, fleas and flies) can also be key vectors of the disease transmission (Dennis et al. 2001; Tarnvik et al. 2003; Oyston et al. 2004; Michelet et al. 2016). Additionally, transfer of the pathogen to humans may occur through direct contact with contaminated animal products (blood, faeces, skin), through inhalation of contaminated air or dust, and by ingesting contaminated food and water (Ohtake et al. 2011).

In order to monitor the presence of *C. burnetii* and *F. tularensis* in environmental samples during epidemiological surveillance, PCR method can be implemented

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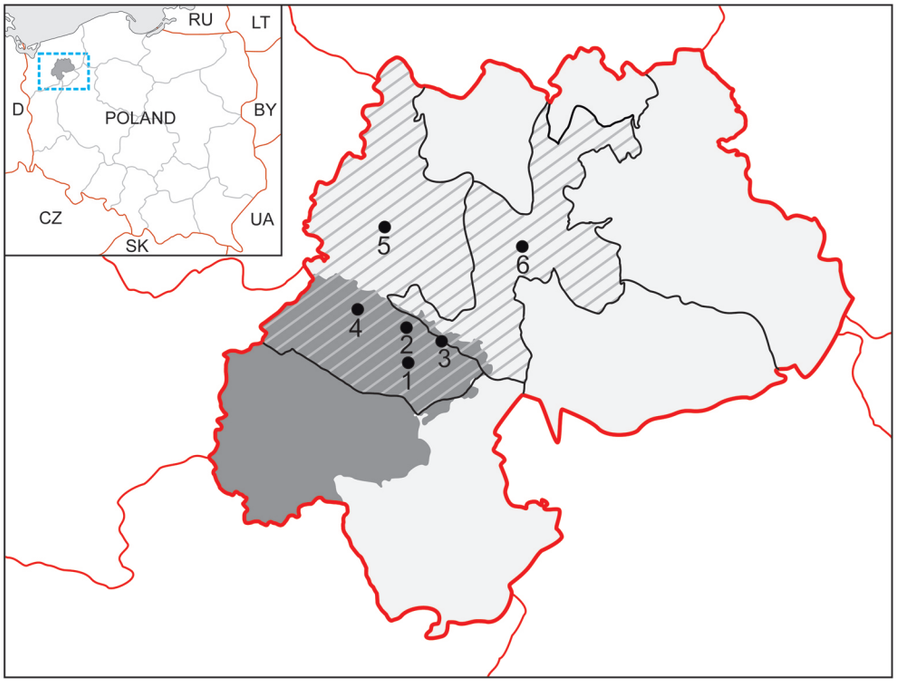


Fig. 1. The locations of sample collection.

Legend: Area marked by the red line: Drawsko County; dark grey slanted striped areas: Drawsko and Złocieniec Districts; grey areas: Drawsko military training ground.

The numbers indicate the areas of sample collections: ticks (1 – Konotop, 2 – Lake Konotop, 3 – Karwice, 4 – Oleszno), and animals (5 – Drawsko-Pomorskie, 6 – Złocieniec).

(Higgins 2000; Emanuel et al. 2003; Seshadri et al. 2003; Fujita et al. 2006; Klee et al. 2006; Petersen et al. 2009; Bielawska-Drózd et al. 2010).

The goal of this study was to determine the occurrence of *C. burnetii* and *F. tularensis* in samples from wild animals and ticks collected from forested areas of Drawsko County including Drawsko military ground (West Pomeranian Voivodeship) using the real-time PCR method.

The wild animals to be sampled were hunted in the years 2016–2017 in the areas, which included the hunting of the following clubs "Cyraneczka" – Drawsko Pomorskie; "Żbik" – Cieszyno near Złocieniec; "Bażant" – Stawno near Złocieniec (Fig. 1). For testing, the samples were collected from internal organs (liver, spleen, heart and lungs). In total, 928 tissue samples were collected from 232 animals from the following species: red deer (140 animals), roe deer (40), and boar (52). The samples, which consisted of mixture of tissues (5–10 g) from four organs examined were suspended in saline solution and homogenized (rotor-stator homogenizer – SHM1, Stuart). Subsequently, 1 ml of the homogenizate was transferred to a new 2 ml test tube and centrifuged for 1 min at room temperature at 6300 rpm. The supernatant (1000 µl) was transferred to a new test tube and centrifuged again for 3 minutes at room temperature at 15 000 rpm. The pellet was resuspended in a lysis buffer, then 2 µl RNase and 10 µl proteinase K were added and incubated for 18 hours at 56°C. Subsequent stages were carried out according to the protocol of GeneMatrix Tissue&Bacterial DNA Purification Kit (EURx Ltd., Poland).

The research also included 1551 ticks collected from five testing sites that were forested areas of Drawsko military training ground (Konotop Encampment, Oleszno and Karwice, Drawa and Konotop Lake) from April to May 2017 (Fig. 1). These ticks were collected by the flagging-dragging method. Tick species were identified using taxonomic keys; the species collected was found to be *Ixodes ricinus*. Ticks were pooled according to the collection site and divided according to sex (adults) and development stages (nymphs): ♀ – female (296 ticks), ♂ – male (250), N – nymph (1005).

80 pooled tick samples (pools) were obtained. Among them, 72 pools contained 20 tick imagos or nymphs (10 pools – ♂, 12 pools – ♀ , 50 pools – N), 3 pools contained 19 ticks (1 pool – ♂, 1 pool – ♀, 1 pool – N), 1 pool contained 17 ticks – ♀, 1 pool contained 13 ticks – ♂, 1 pool contained 12 ticks – ♀, 1 pool contained 7 ticks – N, 1 pool contained 5 ticks – N. In Drawa location pools were as follows: 211 (♀ – 2 pools/20 individuals, 1 pool/19 individuals; ♂ – 2 pools/20 individuals, 1 pool/12 individuals; N – 5 pools/20 individuals), in Karwice location the following samples were obtained:

460 (♀ – 5 pools/20 individuals; ♂ – 4 pools/20 individuals; N – 14 pools/20 individuals), in Lake Konotop location: 377 (♀ – 4 pools/20 individuals, 1 pool/13 individuals; ♂ – 3 pools/20 individuals, 1 pool/19 individuals; N – 10 pools/20 individuals, 1 pool/5 individuals), in Konotop location: 117 (♀ – 1 pool/17 individuals; ♂ – 1 pool/20 individuals; N – 4 pools/20 individuals), in Oleszno location: 386 (♀ – 1 pool/20 individuals, 1 pool/7 individuals; ♂ – 1 pool/19 individual, N – 17 pools/20 individuals) were collected, respectively. The pooled tick samples were placed in 2 ml test tubes with 300 µl of ethanol (70%) inside and left for 15 minutes (stirred several times). The alcohol was removed, and the ticks were rinsed with deionized water (300 µl). The residues of water were removed with blotting paper and the samples were placed in liquid nitrogen for 10 minutes. The ticks were then homogenized (mechanically, in a mortar) (Halos et al. 2004; Rodriguez et al. 2014; Jose et al. 2017). Following that, 1 ml of deionized water was added, and the samples were frozen at –80°C for further analyses. To isolate the genetic material, 200 µl of the homogenized liquid was used. The material was centrifuged at 15 000 rpm for 3 minutes at room temperature. The pellet was resuspended in lysis buffer LyseT, 2 µl RNase and 10 µl proteinase K were added and the material was incubated for 12 hours at the temperature of 56°C. Subsequent stages were carried out according to the protocol by GeneMatrix Tissue&Bacterial DNA Purification Kit (EURx Ltd., Poland).

Screening tests were performed by the real-time PCR method using a *C. burnetii* – specific multicopy insertion sequence *IS1111*$^{+}$ (transposase gene) and the outer membrane coding sequences: *fopA* and *tul4* for *F. tularensis* (these sequences confirmed the presence of *F. tularensis* in ticks). The oligonucleotides used in the reactions are presented in Table I.

The oligonucleotides were synthesized by Genomed S.A. (Poland). The reactions for both pathogens were conducted using LightCycler 2.0 instrument (Roche, Germany) according to the following thermal profile: initial denaturation at 95°C for 10 minutes; 40 cycles (95°C for 15 seconds, 60°C for 30 seconds); 40°C for 30 seconds. DNA extracted from *C. burnetii* Nine Mile and *F. tularensis* subsp. *holarctica* strain Kodar were used as positive controls. The test samples were positive when the cycle threshold $C_t$ was lower than 36.

The prevalence of infected ticks in pools was analyzed based on the number of individuals. Statistical analyses to calculate estimated prevalence for fixed and variable pool sizes were performed with EpiTools (http://www.ausvet.com.au; Andreassen et al. 2012; Ndeereh et al. 2017).

Q fever research in Poland are based on immunological status of both domestic and wild-living animals (Niemczuk et al. 2011). Only few studies involved molecular analyses of clinical or environmental material (arthropods) (Tylewska et al. 1996; Szymańska et al. 2013; Bielawska-Drózd et al. 2014; Bielawska-Drózd et al. 2016). Seven wild-living animals (three boars, three stags and one roe deer) were found positive for *IS1111*$^{+}$, characteristic of *C. burnetii* (3%). In the light of other studies, the positive results obtained in this research are similar or even lower when compared to the results by others: 0.7% in red deers (Smetanova et al. 2016), 4.3% in wild boars 5.1% in roe deers and 9.1% (European hares) (Astobiza et al. 2011).

In this study any characteristic sequences were detected in testing for the presence of *F. tularensis* DNA in wild-living animals. Despite a large proportion of positive results obtained in ELISA by others: 3.5% (Al Dahouk et al. 2005), 7.5% (Kuehn et al. 2013), 7.4% (Otto et al. 2014) or even 15–30% (Taussing and Landau 2008), in this study the biological agent remains undetected in animal tissues.

Epidemiological situation of Q fever and tularemia in Poland as well as worldwide seems to be stable. Although Q fever and tularemia outbreaks have been registered almost all around the world, the numbers of infections are still low, but Scandinavian countries, Hungary and Czech Republic in relation to tularemia (Bielawska-Drózd et al. 2013; ECDC 2016). Most

Table I
The oligonucleotides used in real-time PCR.

| | *C. burnetii* | *F. tularensis* | |
|---|---|---|---|
| | *IS1111*$^{+}$ (Klee et al. 2006) | *fopA* (Emmanuel et al. 2003) | *tul4* (Fujita et al. 2006) |
| Forward primer | 5'-GTCTTAAGGTGGGCTGCGT G-3' | 5'-AACAATGGCACCTAGTAAT ATTTCTGG-3' | 5'-ATTACAATGGCAGGCTCC AGA-3' |
| Reverse primer | 5'-CCCCGAATCTCATTGATC AGC-3' | 5'-CCACCAAAGAACCATGTT AAACC-3' | 5'-TGCCCAAGTTTTATCGTTC TTCT-3' |
| Probe | 5'-FAM-AGCGAACCATTGGTATC GGACGTTT-TAMRA-TATGG -Pho-3' | 5'-FAM-TGGCAGAGCGGGTACT AACATGATTGGT-TAMRA-3' | 5'-FAM TCCTAAGTGCCATGAT ACAAGCTTCCCAATTACTAAG -BHQ1-3' |

Table II
The estimated prevalence of positive ticks pools for *C. burnetii* and *F. tularensis*.

| Site | Number of pools | PoolSize | Number of positive pools for *C. burnetii* | Number of positive pools for *F. tularensis* | Number of individuals | Total number of individuals | Estimated prevalence (%)[1] *C. burnetii* | Estimated prevalence (%)[1] *F. tularensis* |
|---|---|---|---|---|---|---|---|---|
| Drawa | 9 | 20 | 1 | 0 | 59 | 211 | 0.5% | 0.49% |
| | 1 | 19 | 0 | 0 | 52 | | | |
| | 1 | 12 | 0 | 1 | 100 | | | |
| Karwice | 23 | 20 | 2 | 0 | 460 | 460 | 0.45% | 0 |
| Lake Konotop | 17 | 20 | 8 | 0 | 340 | 377 | 3.1% | 0 |
| | 1 | 19 | 0 | 0 | 19 | | | |
| | 1 | 13 | 0 | 0 | 13 | | | |
| | 1 | 5 | 1 | 0 | 5 | | | |
| Konotop | 5 | 20 | 2 | 0 | 100 | 117 | 3.45% | 0 |
| | 1 | 17 | 1 | 0 | 17 | | | |
| Oleszno | 18 | 20 | 2 | 0 | 360 | 386 | 0.55% | 0 |
| | 1 | 19 | 0 | 0 | 19 | | | |
| | 1 | 7 | 0 | 0 | 7 | | | |

outbreaks of tularemia and Q-fever have been in wildlife species. In Germany between 2002 and 2016, 10 clusters of tularemia were reported. A serological study in various wildlife species in Brandenburg revelated a total of 101/1353 positive sera (7.5%) of foxes, raccoon dogs, and wild boars (Faber et al. 2018). The reports from Germany between 1992 and 2012 showed that 2.4% of dead wild European rabbits were positive for *F. tularensis*. In Austria, Bulgaria, Germany, Hungary, Kosovo, Slovakia and Sweden small rodents were examined and the detection rate of *F. tularensis* varied from 0.7% to 20.8%. In Austria, the bacteria was detected in 1.3% of the hunter red foxes. In Portugal, 212 migratory shore of various species were tested for bacteria, which resulted in identification of *F. tularensis* (Hestvik et al. 2015). Q fever epidemics and epizooties in 1948–2004 were registered in the region of the Balkan Peninsula (Hukici et al. 2010). Serum samples from 464 wild rabbits were collected and analyzed from European wild rabbits in Spain, Portugal and Chafarinas Islands during the time period 2003–2013. Seroprevalence in wild rabbit populations ranged from 6.7% to 81.3%. European rabbits can also be reservoirs of *C. burnetii* (González-Barrio et al. 2015).

The estimated prevalence of *C. burnetii* in pools of ticks ranged from 0.45% to 3.45% for the various locations. The estimated prevalence in the Lake Konotop and Konotop areas showed significantly higher value than in the other sites examined ($p$-value 0.00348). These results correspond with the results that have been already reported in Poland and in other countries e.g. Senegal, Netherlands, Iran, Slovakia, Hungary, Spain, Germany (Tylewska and Chmielewski 1996; Spitalska et al. 2003; Toledo et al. 2009; Mediannikov et al. 2010; Fard and Khalili 2011; Hilderbrandt et al. 2011; Sprong et al. 2012; Bielawska-Drózd et al. 2016). Moreover, in the current study *fopA* and *tul4* positive results (0.49%) for *F. tularensis* were found only in one location – Drawa (Table II). Such a small proportion of ticks infected with *F. tularensis* (0.2% to 1.4%) was also found by other studies conducted in Poland, France, Germany and Portugal (Franke et al. 2010; Lopes de Carvalho et al. 2010; Reis et al. 2011; Wójcik-Fatla et al. 2015). Slightly higher proportions: 1.98% and 3.8% were detected by Zhang et al. (2008) and Tomanović et al. (2009), respectively. However, according to the newest research, the two genes *fopA* and *tul4* can also be present in *Francisella*-like endosymbionts (FLEs). Screening research conducted in Portugal by Lopes de Carvalho et al. (2010) demonstrated that no less than 32% ticks of *Dermacentor reticulatus* had the *tul4* gene. In subsequent research by Michelet et al. (2016) FLEs were detected in 86% of *D. reticulatus* as it was shown by the presence of the *fopA* gene. Therefore, indisputable presence of *F. tularensis* may be only confirmed when both genes *tul4* and *fopA* are present in the sample tested, while FLEs are supposed when only one of the markers is detected. Due to the highest prevalence of FLEs in *D. reticulatus* the simultaneous monitoring of the presence of both sequences is necessary to exclude FLEs. Although the research presented here did not encompass *D. reticulatus* but only *I. ricinus* species, one has pay attention to *D. reticulatus* since it can constitute an unquantified FLEs reservoir in Poland. Therefore, future studies ought to comprise separate analyses for this species and consider its specifics and seasonality.

The results obtained in this work correlate with the data found by other researchers. However, despite the results did not present an increase in the proportion of the ticks infected, it is worth to continue studies by Formińska et al. (2015) and Chmielewski et al. (2010) and to examine more numerous tick species and biological agents that can exist in arthropods and constitute a significant threat to human health and lives. It is also advisable to extend the scope of research to include other wild-living animal species as potential reservoirs of zoonotic pathogens.

Additionally, this research demonstrates the useful molecular tool for the detection of *F. tularensis* and *C. burnetii* during natural tularemia and Q fever outbreaks.

**Authors' contributions**

ABD and PC designed the study and processed the data. PC, BWS and DZ completed the analysis. PZ and AZ analyzed the reservoirs and biological samples, ABD, PC, PG participated in the manuscript preparation. All authors read, completed and approved the final manuscript.

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**Conflict of interest**

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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**E**

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**F**

Farshadzadeh Zahra (Iran), Feβler Andrea T.T. (Germany), Flores Julio Parra (Chile)

**G**

Gcebe Nomakorinte (South Africa), Gibson Brian (Finland), Gościniak Grażyna (Poland), Guzek Aneta (Poland), Guz-Regener Katarzyna (Poland)

**H**

Haddix Pryce L. (USA), He Yinfeng (China), Heczko Piotr (Poland), Hoffman Monika (Poland), Hu Lijun (USA)

**J**

Jain Amita (India), Jaroszuk-Ściseł Jadwiga (Poland), Jarosz-Wilkołazka Anna (Poland), Junka Feliks (Poland)

**K**

Kaliszewski Miron (Poland), Kalyanasundaram Dinesh (India), Kazimirova Maria (Slovakia), Kędziora Anna (Poland), Klewicka Elżbieta (Poland), Kmush Brittany L. (USA), Koch Eckhard (Germany), Kochan Piotr (Poland), Korzeniewska-Kosela Maria (Poland), Kosikowska Urszula (Poland), Kovacs Renato (Hungary), Krowarsch Daniel (Poland), Kuczyńska-Styczyńska Alina (Poland)

**L**

Latovetsky Olga (USA), Laudy Agnieszka E. (Poland), Lee I Learn-Han (Malaysia), Lehner Angelika (Switzerland), Lei Bo (Hong Kong), Litwińska Bogumiła (Poland), Liu Wenghong (China), Lo Horng-Ren (Taiwan), Long Longfei (China), Ławniczak Łukasz (Poland)

**M**

Ma Shaohui (China), Madut Nuol A. (Uganda), Majewska Małgorzata (Poland), Malm Anna (Poland), Marcinkiewicz Janusz (Poland), Markiewicz Lidia (Poland), Martins Roberto (Portugal), McRose Darcy (USA), Mielczarek Agnieszka (Poland), Mikucki Dawid (Poland), Millar Beverly C. (United Kingdom), Miyoshi Shin-Ichi (Japan), Mora-Montes Hector Manuel (Mexico), Murad Yanal (Pakistan)

**N**

Nawani Neeli N. (India), Nawrocka Justyna (Poland), Nawrot Urszula (Poland), Neher Deborah (USA), Netala Vasudeva Reddy (India), Newman David J. (USA)

**O**

Ogórek Rafał (Poland), Olender Alina M. (Poland), Ouédraogo Samiratou (Canada)

**P**

Paściak Mariola (Poland), Pereira Flaviane Gomez (Brazil), Pierre Joseph Francis (USA), Platonov Aleksander (Russia), Podsiadły Edyta (Poland), Pugazhendhi Arivalagan (Vietnam), Rastawicki Waldemar (Poland), Ronai Zsuzsanna (Hungary), Różalska Barbara (Poland)

**S**

Santara Sumit Sen (USA), Saune Karine (France), Sayan Murat (Turkey), Schönning Kristian (Denmark), Sergi Consolato (Canada), Sgibnew Andrey (Russia), Sipailienė Aušra (Lithuania), Staniszewska Monika (Poland), Stączek Paweł (Poland), Stępińska Małgorzata (Poland), Strus Magdalena (Poland), Suhartono Maggie (Indonesia), Sultana Taranum (Canada), Szewczyk Eligia (Poland)

**T**

Tall Ben D. (USA), Trincone Antonio (Italy)

**V**

Verna Pradeep (India)

**W**

Waditee-Sirisattha Rungaroon (Thailand), Wałecka-Zaharska Ewa (Poland), Washington Michael A. (USA), Wołko Łukasz (Poland), Wójkowska-Mach Jadwiga (Poland)

**X**

Xu Zhenbo (China)

**Y**

Yang Jin-Long (China), Yano Yoshihiko (Japan), Yokota Shin-ichi (Japan)

**Z**

Zasada Aleksandra (Poland), Zdybicka-Barabas Agnieszka (Poland), Ziora Zyta M.M. (Australia)

Polish Journal of Microbiology
2018, Vol. 67, No 4, 543–548

## INFORMACJE Z POLSKIEGO TOWARZYSTWA MIKROBIOLOGÓW
## 06.12.2018

KONFERENCJA POD PATRONATEM PTM

**Konferencja międzynarodowa:**

**8 Międzynarodowa Konferencja Weiglowska**
**Łódź, 26–28.06.2019**


Konferencja organizowana jest przez
Instytut Biologii Medycznej PAN, Uniwersytet Łódzki
oraz Uniwersytet Medyczny w Łodzi

The planned Conference is the eighth conference to commemorate the outstanding Polish microbiologist Prof. Rudolf Weigl, the creator of the first effective vaccine against typhus. The conference is devoted to selected medical, microbiological and biotechnology issues. As part of the conference, thematic sessions will be devoted to (i) gastrointestinal diseases of various etiologies, (ii) skin diseases, (iii) and ophthalmologic cancerous and autoimmune diseases. In addition, there will be (iv) a molecular microbiology session focused on recent developments in microbial virulence, cell metabolism, signal transduction, bacterial biofilm studies and single cell analyzes. The last session (v) will be dedicated to microorganisms in industrial and medical biotechnology. The conference will be a unique platform for meetings and discussions for researchers dealing with the molecular basis of selected diseases, for microbiologists investigating these processes at the level of virulence factor, but also for clinicians involved in everyday diagnostics and treatment of diseases. The conference will also create a unique opportunity to familiarize participants of the session, including students and doctoral students, with different perspectives of research on the same processes, from the human body to pathogenic or saprophytic microorganisms and their mutual interactions.

http://grupamedica.pl/8th-international-weigl-conference-26-28-06-2019/

www.twitter/grupamedica ; https://web.facebook.com/8thweiglconferene/

KONFERENCJA POD PATRONATEM PTM

**VII Konferencja Ogólnopolska:**
**Mikrobiologia Farmaceutyczna 2019**
**Gdańsk, 23–25 maja 2019 r.**

Konferencja jest kontynuacją rozpoczętego w 2009 roku cyklu spotkań naukowo szkoleniowych, którego celem jest stworzenie Forum Specjalistów zainteresowanych mikrobiologią farmaceutyczną, służącego wymianie wiedzy, poglądów i doświadczeń w obszarach oceny i zapewnienia jakości, badań mikrobiologicznych oraz bezpieczeństwa mikrobiologicznego produktów leczniczych.

Na spotkaniach dążymy do inicjowania szeroko pojętej współpracy między mikrobiologami pracującymi głównie w przemyśle farmaceutycznym, ale też w spożywczym i kosmetycznym.

Patronat Polskiego Towarzystwa Mikrobiologicznego jest dla nas bardzo ważny, to PTM jest organizacją umożliwiającą współpracę, rozwój i kreowanie nowych rozwiązań w dziedzinie Mikrobiologii, a utworzenie sekcji Mikrobiologii Farmaceutycznej umożliwia ciągłe współdziałanie i rozwój Członków.

Podczas naszych konferencji promujemy przynależność do PTM i członkowie PTM już od poprzedniej VI konferencji Mikrobiologia Farmaceutyczna mają zniżkę 100 zł w opłacie konferencyjnej. W konferencjach bierze udział zwykle 80–100 osób z przedsiębiorstw farmaceutycznych, laboratoriów kontrolnych i uczelni.

Kontakt do Organizatora:
TRANSpharmacia, Pani Barbara Kawałko-Myślińska,
barbara.myslinska@transpharmacia.pl, ul. Bacewiczówny 2/42, 02-786 Warszawa

**Poniżej w punktach, w formie skrótowej przedstawiono omawiane sprawy na wirtualnych zebraniach Prezydium ZG PTM oraz spotkaniach z udziałem członków Prezydium.**

**1.** W dniach 14–15 września 2018 roku w Poznaniu odbył się II Kongresu Towarzystw Naukowych pod tytułem „Rola towarzystw naukowych w rozwoju świadomości obywatelskiej i kulturowej". Kongres został zorganizowany przez Radę Towarzystw Naukowych PAN, Poznańskie Towarzystwo Przyjaciół Nauk, Polską Akademię Umiejętności oraz Towarzystwo Naukowe Płockie pod Patronatem Ministra Nauki i Szkolnictwa Wyższego oraz Ministra Kultury i Dziedzictwa Narodowego.
Uczestniczyło w nim ponad 200 osób reprezentujących ponad 450-tysięczną społeczność członków towarzystw naukowych i stowarzyszeń naukowo-technicznych.
Polskie Towarzystwo Mikrobiologów reprezentowała Pani prof. dr hab. n. med. Ewa Augustynowicz-Kopeć, Wiceprezes PTM.
Celem Kongresu było przedstawienie wkładu społecznego ruchu naukowego w rozwój świadomości narodowej, obywatelskiej i kulturowej w okresie od odzyskania niepodległości przez Polskę do chwili obecnej, a także dyskusja nad aktualnym miejscem społecznego ruchu naukowego w systemie nauki w Polsce. W ramach Kongresu były poruszane następujące zagadnienia:

- Rozwój i wkład towarzystw naukowych w kształtowanie świadomości narodowej i obywatelskiej na przestrzeni wieków.
- Ocena wkładu towarzystw naukowych w rozwój społeczeństwa obywatelskiego,
- Rozwój i wkład towarzystw naukowych w kształtowanie kultury polskiej w okresie od odzyskania niepodległości Polski.
- Wpływ towarzystw naukowych na rozwój nauki w Polsce w okresie od odzyskania niepodległości Polski.
- Wpływ towarzystw naukowych na rozwój społeczno-ekonomiczny i rozwój regionów w okresie od odzyskania niepodległości Polski.
- Wpływ towarzystw naukowych na rozwój techniki i gospodarki w Polsce i w poszczególnych regionach od odzyskania niepodległości Polski.
- Ocena wkładu towarzystw naukowych w upowszechnianie nauki w Polsce i rozwój nowoczesnych form upowszechniania nauki (festiwale nauki, pikniki naukowe).

- Aktualny system dofinansowania społecznego ruchu naukowego w Polsce ze środków przeznaczanych na naukę.
- Sytuacja prawna społecznego ruchu naukowego w aktualnym systemie nauki w Polsce.

W referatach i komunikatach, wygłoszonych na sesjach problemowych Kongresu, przedstawiono historię, dotychczasową działalność i dorobek poszczególnych towarzystw i stowarzyszeń naukowych i naukowo-zawodowych oraz stowarzyszeń naukowo-technicznych NOT. Podsumowaniem było przyjęcie Uchwały II Kongresu Towarzystw Naukowych, w której stwierdzono, że:

– towarzystwa naukowe i stowarzyszenia naukowo-techniczne odegrały doniosłą rolę w czasach zaborów w dziejach niepodległego Państwa Polskiego i są integralną częścią nauki polskiej, organizując społeczny ruch naukowy i budując społeczeństwo obywatelskie oraz podmiotami życia publicznego i ze względu na swoje znaczenie dla społeczeństwa powinny znaleźć należne miejsce w systemie prawnym w kraju.

– towarzystwa naukowe powinny być wspierane przez budżet państwa oraz budżety samorządowe ze względu na ich znaczenie dla rozwoju społeczeństwa. Kongres uznał, że przy stosunkowo niskim rozumieniu znaczenia nauki dla rozwoju naszego kraju należy wzmocnić rolę publikacji i innych działań odnoszących się do popularyzacji wiedzy i wyników badań. Podniesienie rangi takiej aktywności powinno następować nie tylko przez wsparcie materialne, ale i przez docenianie jej we wszystkich coraz bardziej sformalizowanych ocenach.

– uczestnicy Kongresu uznali, że bez zmiany polityki państwa w stosunku do społecznego ruchu naukowego spiętrzać się będą bariery rozwoju naukowego, kulturalnego, gospodarczego i społecznego Polski oraz zniweczony zostanie ogromny dorobek towarzystw naukowych.

**2.** W dniu 24.10.2018 r. odbyło się spotkanie Prezesa i Sekretarz PTM z Redaktorami Naczelnymi i Sekretarzami czasopism PM i PJM oraz z Panem Dawidem Ceculą Dyrektorem firmy Exeley Inc. wydającej *on-line* kwartalnik Polish Journal of Micorbiology https://www.exeley.com/journal/polish_journal_of_microbiology. Omówiono sprawy związane z usprawnieniem działania systemu Editorial Manager oraz współprace redakcji PJM z Exeley Inc. Ponadto rozpoczęto rozmowy nad włączeniem od 2019 r. czasopisma Postępy Mikrobiologii do procesu edytorskiego prowadzonego przez powyższą firmę wydawnicza, co powinno zaowocować zwiększeniem cytowalności i wartości współczynnika Impact Factor.

**3.** Rozpoczęliśmy, we współpracy z firmą tłumaczy z Lublina, proces tłumaczenia na język angielski artykułów przesłanych do numerów 3 i 4 z 2018 r. Postępów Mikrobiologii. Działalność ta jest możliwa, dzięki otrzymaniu dofinansowania z Ministerstwa Nauki i Szkolnictwa Wyższego w ramach Działalności Upowszechniającej Naukę (Umowa nr 659/P-DUN/2018). Biorąc pod uwagę, że dofinansowanie na tłumaczenie otrzymamy także w przyszłym roku, planujemy publikować artykuły w obu językach – polskim i angielskim w zeszytach PM w 2019 r. Artykuły z PM w języku angielskim będą mogły być czytane i cytowane nie tylko przez polskich czytelników. Taka działalność również ma na celu zwiększenie cytowalności i wartości współczynnika Impact Factor.

**4.** Prezydium ZG PTM podjęło pozytywną decyzję w sprawie objęcia patronatem czasopisma „Zakażenia XXI wieku" – oficjalnego, recenzowanego czasopisma Polskiego Towarzystwa Zakażeń Szpitalnych. Patronat ma polegać na zamieszczeniu linku do czasopisma na stronie PTM, wsparciu merytorycznym czasopisma przez PTM oraz podawaniu propozycji artykułów. W zamian, czasopismo „Zakażenia XXI wieku" będzie informować o organizowanych przez PTM konferencjach i seminariach, w czasopiśmie oraz na stronie internetowej. **Uchwała nr 34–2018.**

**5.** Prezydium ZG PTM podjęło pozytywną decyzję w sprawie objęcia patronatem konferencji „8th International Weigl Conference", organizowanej przez Instytut Biologii Medycznej PAN, Uniwersytet Łódzki oraz Uniwersytet Medyczny w Łodzi, w terminie 26–28.06.2019 r., oraz poparcia wniosku Komitetu Organizacyjnego Konferencji o dofinansowanie konferencji przez FEMS (w przypadku przyznania środków przez FEMS, PTM również dofinansuje konferencję w kwocie 250 Euro). **Uchwała nr 35–2018.**

**6.** Prezydium ZG PTM podjęło pozytywną decyzję w sprawie objęcia patronatem VII Konferencji Mikrobiologia Farmaceutyczna organizowanej w Gdańsku w dniach 23–25 maja 2019 r. Bez kosztów PTM. **Uchwała nr 36–2018.**

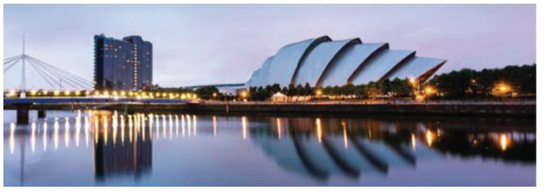
**7.** Do Nagrody Naukowej im. prof. Edmunda Mikulaszka Polskiego Towarzystwa Mikrobiologów zgłosiło się tylko 2 kandydatów. Komisja Konkursowej pod przewodnictwem Pani prof. dr hab. Stefanii Giedrys-Kalemby po przeprowadzeniu dyskusji drogą e-mailową, proponuje przyznać dwie równorzędne nagrody II stopnia, po 3.000 zł: mgr inż. biot. Pawłowi Kwiatkowskiemu za pracę: P. Kwiatkowski i wsp.: The effect of fennel essential oil in combination with antibiotics on *Staphylococcus aureus* isolated from carriers. Burns, 2017, 43: 1544-1551 ($IF_{2017}$ – 2.134, MNISW – 25 pkt).
dr inż. Wojciechowi Smułek za pracę: W. Smułek i wsp.: *Sapindus* saponins' impact on hydrocarbon biodegradation by bacteria strains after short- and long-term contact with pollutant. Colloids and Surfaces B: Biointerfaces 2016, 142: 207–213 ($IF_{2016}$ – 3.887, MNISW – 35 pkt).
Prezydium Zarządu Głównego PTM podejmie w powyższej sprawie stosowną uchwałę.

**8.** Informujemy, że 3 Członków Wspierających PTM – firmy: HCS Europe-Hygiene & Cleaning Solutions, Ecolab Sp. z o.o. oraz Merck Sp.z o.o. uiściły już swoje składki członkowskie za 2018 rok, za co bardzo dziękujemy.

**9.** **Uchwałą nr 33–2018** Prezydium ZG PTM przyjęto do PTM siedmiu nowych Członków Zwyczajnych.

**10**. Dosyć opieszale posuwa się sprawa opracowania regulaminu wydatkowania i rozliczania funduszy, które zaplanowano przyznać Oddziałom Terenowym PTM w kwocie równoważnej 10% opłat członkowskich wnoszonych przez członków PTM z danego Oddziału. **Uchwałą ZG PTM nr 23–2018** z dnia 19.03.2018 r. powołana została 3 osobowa Komisja do opracowania tego regulaminu. W dniu 13.10.2018 r. Komisja przesłała do sekretariatu ZG PTM pierwszy projekt roboczy regulaminu. Ostateczny projekt regulaminu musi uzyskać aprobatę Księgowej PTM, Prezydium ZG PTM oraz całego ZG PTM. Oczekujemy na kolejne wersje projektu uwzględniające zgłaszane do Komisji uwagi. Jeżeli projekt nie uzyska wstępnych akceptacji, nie będzie poddany głosowaniu na dorocznym zebraniu ZG PTM planowanym w marcu w 2019 r. i środki nie będą mogły być udostępnione Oddziałom.

**11.** Przypominamy o 8 Kongresie Europejskich Mikrobiologów organizowanym przez FEMS 7–11 lipca 2019 r. w Glasgow, http://fems2019.org/. Termin składania wniosków przez młodych naukowców o wsparcie uczestnictwa w Kongresie – 15 stycznia 2019 r. Ostateczny termin nadsyłania abstraktów na Kongres, to również 15 stycznia 2019 r.



FEMS 2019

8th Congress of European Microbiologists

7-11 July 2019 | Glasgow, Scotland

**12.** Przypominamy terminy składania wniosków o granty FEMS: Research and Training Grants – 01.01.2019 r., Congress Attendance Grants – 15.01.2019 r.

**13.** Na dzień 5.12.2018 r. Polskie Towarzystwo Mikrobiologów liczy 957 członków. Liczba członków PTM w Oddziałach Terenowych wynosi odpowiednio: OT Warszawa – 198, OT Kraków – 131, OT Katowice – 99, OT Bydgoszcz – 78, OT Poznań – 76, OT Szczecin – 66, OT Lublin – 62, OT Gdańsk – 57, OT Łódź – 47, OT Wrocław – 35, OT Białystok – 33, OT Olsztyn – 31, OT Kielce – 24, OT Puławy – 20.
Składki za rok 2017 r. **(2018 r.)** zapłaciła następująca liczba członków danego oddziału: OT Warszawa – 175 **(112)**, OT Kraków – 124 **(96)**, OT Katowice – 93 **(72)**, OT Bydgoszcz – 75 **(47)**, OT Poznań – 75 **(53)**, OT Szczecin – 62 **(39)**, OT Lublin – 60 **(46)**, OT Gdańsk – 53 **(39)**, OT Łódź – 45 **(30)**, OT Wrocław – 31 **(25)**, OT Olsztyn – 29 **(26)**, OT Białystok – 27 **(21)**, OT Kielce –19 **(15)**, OT Puławy –18 **(11).**
**Członkowie PTM, którzy nie opłacili jeszcze składki za 2018 r. proszeni są o pilne jej uzupełnienie.**

***W związku ze zbliżającymi się Świętami Bożego Narodzenia i Nowego Roku 2019, przekazujemy Państwu najserdeczniejsze życzenia zdrowych, pogodnych i szczęśliwych Świąt oraz wielu sukcesów, pomyślności i spokoju w nadchodzącym roku.***

SEKRETARZ
Polskiego Towarzystwa Mikrobiologów
*dr hab. n. farm. Agnieszka E. Laudy*

PREZES
Polskiego Towarzystwa Mikrobiologów
*prof. dr hab. Stefan Tyski*

## CZŁONKOWIE WSPIERAJĄCY PTM

**Członek Wspierający PTM – Złoty**
**od 27.03.2017 r.**

HCS Europe – Hygiene & Cleaning Solutions
ul. Warszawska 9a, 32-086 Węgrzce k. Krakowa
tel. (12) 414 00 60, 506 184 673, fax (12) 414 00 66
www.hcseurope.pl

Firma projektuje profesjonalne systemy utrzymania czystości i higieny dla klientów o szczególnych wymaganiach higienicznych, m.in. kompleksowe systemy mycia, dezynfekcji, osuszania rąk dla pracowników służby zdrowia, preparaty do dezynfekcji powierzchni dla służby zdrowia, systemy sterylizacji narzędzi.

Członek Wspierający PTM – Srebrny
**od 07.06.2017 r.**

Aesculap Chifa Sp. z o.o.
ul. Tysiąclecia 14
64-300 Nowy Tomyśl
tel. (61) 44 20 100, fax (61) 44 23 936
www.chifa.com.pl

Aesculap Chifa Sp. z o.o. jest członkiem grupy B. Braun, jednej z wiodących na świecie firm medycznych, produkującej i dystrybuującej miedzy innymi preparaty do antyseptyki rąk, skóry, błon śluzowych, do mycia i dezynfekcji wyrobów medycznych oraz powierzchni.

Członek Wspierający PTM – Srebrny
**od 12.09.2017 r.**

Firma Ecolab Sp. z o.o. zapewnia: najlepszą ochronę środowiska pracy przed patogenami powodującymi zakażenia podczas leczenia pacjentów, bezpieczeństwo i wygodę personelu, funkcjonalność posiadanego sprzętu i urządzeń. Firma jest partnerem dla przemysłów farmaceutycznego, biotechnologicznego i kosmetycznego.

**Członek Wspierający PTM – Srebrny**
**od 12.12.2017 r.**

Od ponad 100 lat siedziba Wodociągów Krakowskich mieści się przy ul. Senatorskiej. Budowę obiektu ukończono w 1913 roku. W 2016 r. do sieci wodociągowej wtłoczono ponad 56 mln $m^3$ wody. Szacuje się, że ponad 99,5% mieszkańców Gminy Miejskiej Kraków posiada możliwość korzystania z istniejącej sieci wodociągowej.

**Członek Wspierający PTM – Zwyczajny**
**od 12.09.2017 r.**

Merck Sp. z o.o. jest częścią międzynarodowej grupy Merck KGaA z siedzibą w Darmstadt, Niemcy i dostarcza na rynek polski od roku 1992 wysokiej jakości produkty farmaceutyczne i chemiczne, w tym podłoża mikrobiologiczne