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Significance of Meningococcal Hyperinvasive Clonal Complexes and their Influence on Vaccines Development

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Abstract

Neisseria meningitidis is a commensal of human nasopharynx and humans are the only known reservoir and host of this bacterium. It is also known as a dangerous and devastating pathogen, and infection with *N. meningitidis* may lead to rapidly progressing septicemia or meningitis. These severe infections, called invasive meningococcal disease (IMD), are one of the major public health threats worldwide. IMD may occur sporadically, but also in outbreaks, epidemics, and pandemics. Most of the IMD cases in the world are caused by isolates of genetically related groups, clonal complexes (CC), including those with special epidemiological significance called hyperinvasive clonal complexes. It is still unknown why some of them may persist for decades, whereas other are quickly replaced and disappear. As a consequence, the epidemiological situation of IMD is variable worldwide and greatly depends on the emergence and widespread of clones belonging to hyperinvasive clonal complexes. Their occurrence has serious implications for health policy, requiring often mass immunization campaigns. Paradoxically, alarming situations caused by hyperinvasive CCs stimulated the development and introduction of new vaccines against meningococci. Despite the unquestionable success of these vaccines, isolates of hyperinvasive clones constitute a permanent public health threat, because they are constantly circulating and able to modify their antigenic profiles to escape the host immune response. Therefore, continuous monitoring of meningococcal isolates including thorough molecular typing is indispensable and fundamental for taking appropriate preventive measures.

Key words: *Neisseria meningitidis*, clonal complex, epidemic, invasive meningococcal disease (IMD), meningococcal epidemiology

Introduction

Neisseria meningitidis (meningococcus, Men) is a gram-negative diplococcus carried as a commensal in the upper respiratory tract (nasopharynx) of an average of 8% to 25% humans who are the only known reservoir and host of this bacterium (Stephens, 2009). It is also known as a dangerous and devastating human pathogen, which can cross the mucosal surface, enter the bloodstream and multiply, leading to rapidly progressing septicemia or translocate across the blood-brain barrier causing meningitis. These severe infections called invasive meningococcal disease (IMD) are one of the major public-health threats worldwide (Stephens *et al.*, 2007).

The basic classification of meningococci is based on structural differences of the polysaccharide capsule, which is the major antigen linked to meningococcal virulence. Twelve serogroups have been distinguished and 6 of them (A, B, C, W, X, Y) are responsible for the majority of IMD worldwide. Meningococcal infections usually develop rapidly and at the beginning

can be difficult to distinguish from other less severe febrile illnesses. General case fatality rate (CFR) is high, 9–12% and up to 40% in septicemia (Rosenstein *et al.*, 2001). Death can occur within a few hours from the first appearance of symptoms, and those who survive may suffer from permanent tissue damage, amputations, deafness and psycho-neurological sequelae. That is why rapid diagnosis, targeted antibiotic therapy and chemoprophylaxis are so crucial (Rosenstein *et al.*, 2001; Stephens, 2009). However, due to the very rapid and dramatic course of infection and often lack of time to administer proper treatment, it seems indisputable that the most effective way to prevent IMD is immunoprophylaxis. Commercially available polysaccharide vaccines protect against infections caused by meningococci of serogroup A, C, W and Y. The polysaccharide of serogroup B (MenB) is poorly immunogenic and homologous to human neural tissue. Consequently, vaccine studies against MenB have been focused on protein antigens and have led to the development of *e.g.* outer membrane vesicles (OMV) vaccines (Caesar

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et al., 2013; Panatto *et al.*, 2013; Vipond *et al.*, 2012) and others like Bexsero[®], recently licensed in Europe (EMA, 2013) and in the USA (FDA, 2015) or Trumenba[®] up to date in the USA only (FDA, 2014).

Meningococcal virulence

Although many components and mechanisms associated with meningococcal virulence have been studied, the pathogenesis of IMD is still not fully understood. There are several bacterial factors like capsule, outer membrane components including *i.e.* pili, lipooligosaccharide (LOS) and outer membrane proteins (OMPs) which are associated with adhesion, transmission and invasion of meningococci. The key factor linked to meningococcal virulence is a polysaccharide capsule determining the serogroup. It has antiadherent properties affecting transmission and protects bacteria from phagocytosis, opsonization and complement-dependent bacteriolysis during invasion of host cells. It is also the major antigen of meningococci (except MenB) leading to a rise of bactericidal antibodies. Taking these into account and the fact that antigens A, C, W and Y have been predominant among meningococci, they have been used in polysaccharide and/or conjugate vaccines. Among other meningococcal factors which promote adherence to host cells, are pili and opacity proteins (*i.e.* Opa, Opc). Lipooligosaccharide is also involved in adherence and is known for its strong endotoxic activity. Porins such as PorA, PorB allow the passage of ions across the cell membrane. They modulate apoptosis and influence host immune response. Because they induce bactericidal antibodies during meningococcal disease, they have been used as vaccine antigens. Additionally, differences in porins composition are the basis of identification of serotype (PorB) and serosubtype (PorA). Proteins associated with iron acquisition *e.g.* FetA are also related to virulence and like porins are the target for bactericidal antibodies. In recent years many other outer membrane proteins have been identified, which are involved in the pathogenesis of IMD. Some of them became already, whereas others are still promising vaccine candidates. (Hill *et al.*, 2010; Tzeng and Stephens, 2000). Expression of the above mentioned components is dependent on many mechanisms. For example, the ability of meningococci to exchange genetic material responsible for capsule synthesis may cause modification of the capsule and change of serogroup, called capsule switching (Swartley, 1997). This property applies also to surface protein antigens like *e.g.* PorA, PorB, FetA (antigenic switch). Other mechanisms lead to on/off expression (phase variability) and concerns *i.e.* the capsule and LPS. Consequently, meningococci can become unrecognizable

by the host and may escape from the immune response (Hill *et al.*, 2010, Tzeng and Stephens, 2000).

It is for sure that the course of infection depends not only on a bacteria's ability to invade, but also is host and environmental dependent. Factors that predispose to IMD are *i.e.* the lack of protective bactericidal antibodies, defects in the terminal complement pathway (C5-C9), the lack of properdin, immune suppression associated with splenectomy, nephritic syndrome or hypogammaglobulinemia. Viral upper respiratory tract infections as well as active and passive smoking are associated with injury of respiratory mucosa, which is a barrier to invasion, therefore its damage increases the risk of bacterial transmission and IMD. Transmission is also simplified by close contact with patient and crowding conditions *e.g.* in dormitory, military base, social events (Rosenstein *et al.*, 2001, Tzeng and Stephens, 2000).

Epidemiology of IMD

IMD may occur sporadically, but also in outbreaks, epidemics, and pandemics. The incidence of IMD is geographically variable and age-specific. The overall incidence is from less than 0.5 cases/100 000 to 1000 cases/100 000 population during epidemics in the sub-Saharan African countries (so-called "meningitis belt") (Halperin *et al.*, 2012). The incidence is the highest in children under 5 (especially in infants), teenagers and young adults (Rosenstein *et al.*, 2001; Stephens, 2009).

Distribution of serogroups varies globally, and has been changing during past decades. Meningococci of serogroups B and C (MenC) have been predominant in Europe, Australia and the Americas. Both have been responsible for sporadic cases, but significantly MenC are related to multiple outbreaks and epidemics whereas MenB to threatening hyperendemic situations/prolonged epidemics. Meningococci of serogroup A (MenA) have been the cause of large, seasonal epidemics in Africa and were also prevailing in Asia while in other continents they are very rare (Halperin *et al.*, 2012; Harrison *et al.*, 2009). In Africa, meningococci of serogroup X have been also reported to cause large outbreaks between 2006 and 2010 (Jafri *et al.*, 2013). Meningococci with capsule antigen Y are responsible for one third of IMD cases in the USA. Interestingly, they were rarely observed in Europe in the past, while recently noteworthy increase in the Scandinavian countries have been observed (Bröker *et al.*, 2014; Harrison *et al.*, 2009). Epidemic potential of serogroup W meningococci (MenW) associated generally with very sporadic IMD, was recognized during two epidemics started in Saudi Arabia during Hajj Pilgrimages and a large epidemic in Burkina Faso (Harrison *et al.*, 2009; Jafri *et al.*, 2013).

Molecular typing

To know and understand the epidemiology of IMD and why some isolates are more virulent than others, profound epidemiological analysis of cases including typing of isolates is needed. In the past, phenotype characteristics based on determination of serogroup: serotype:serosubtype (defined by capsule:PorB:PorA, respectively *e.g.* C:2a:P1.5,2) was used but because of lack, poor or masked expression of surface antigens, not all isolates could be typed. In contrast, molecular methods based on PCR and DNA sequencing, provide typing data always when a gene is present, even without protein expression. Additionally, most DNA-based methods can be directly used on clinical samples, when a culture is negative due to *e.g.* early antibiotic treatment. These techniques have higher discriminatory power and generally enable distinction between isolates responsible for outbreaks and sporadic cases. Currently, a widely used method in molecular typing is multilocus sequence typing (MLST). This technique was introduced for the first time in 1998, for meningococci and later adapted for other bacterial species. MLST is based on the sequencing of internal fragments of seven house-keeping genes whose alleles combinations determine sequence type (ST). Among meningococcal numerous STs, there are groups of epidemiologically significant and related STs called clonal complexes (CC). Each CC has one central ST, with the exception of 41/44CC which has two. Isolates that have at least four out of seven loci identical with central ST are considered to be related and form CC (Brehony *et al.*, 2007; Maiden *et al.*, 1998). At present, more than 11 000 sequence types are determined and grouped into 46 clonal complexes whereas these without relatedness to other STs were designated as singletons (<http://pubmlst.org/neisseria/>, Brehony *et al.*, 2007). Despite large variability, there is a limited number of CCs characterised by increased propensity to cause invasive disease or epidemics. They are called hyperinvasive clonal complexes and are responsible for the majority of meningococcal infection in the world. Despite their deep analysis including genome sequencing in comparison to carriage strains, the reasons of their superiority and predominance are still not fully understood (Schoen *et al.*, 2014)

The most common hyperinvasive clonal complexes responsible for IMD – historical background and the present status

Some hyperinvasive CCs have been persistent for many decades, other emerged recently or were prevalent in the past and nowadays are observed very rarely. In spite of this variability, among all hyperinvasive

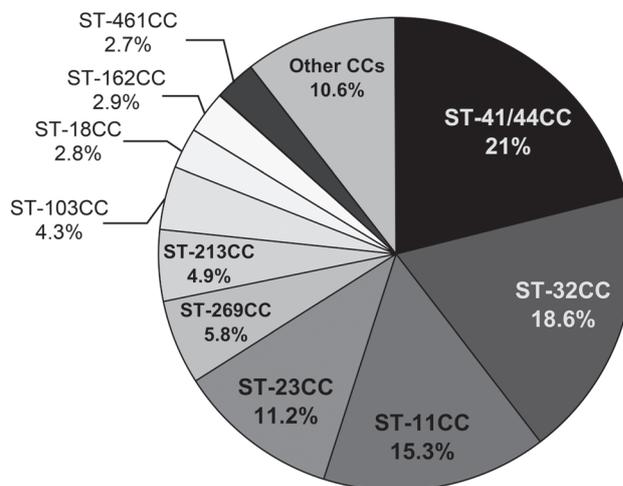


Fig. 1. Distribution of MLST clonal complexes (CCs) among *N. meningitidis* isolates responsible for IMD in Europe in 2011 (in accordance to Whittaker, 2013).

CCs the most frequently mentioned in the literature and overrepresented among meningococci are those belonging to ST-5CC, ST-32CC, ST-41/44CC and ST-11CC. European data confirmed that in 2011 meningococci of ST-41/44CC were the most common, followed by ST-32CC and ST-11CC and their percentage was estimated as high as 21%, 18.6% and 15.3%, respectively (Fig. 1.; Whittaker, 2013). Isolates of the four mentioned CCs have been associated in the past with large epidemics, numerous outbreaks, temporary/geographical incidence increase or long-lasting hyperendemic situations/prolonged epidemics. Their emergence and expansion required public health investigations and targeted interventions, like mass vaccination campaigns using available vaccines but also had an indisputable influence on the construction of new ones. (Table I) (Read, 2014; Watkins and Maiden, 2012).

ST-5CC

ST-5 clonal complex is represented by meningococci of serogroup A. Isolates belonging to this CC were in the past the cause of a few pandemics and since 80s have been predominant in Asia and especially in the “meningitis belt” comprising countries in Sahelian and sub-Saharan Africa. These isolates have been devastating for Africa, being responsible for thousands of IMD cases during yearly epidemics starting with the dry season and cyclical large epidemics occurring every 5–10 years; for example in 1996 there were 150 000 cases and 16 000 deaths reported (Caugant and Nicolas, 2007; Achtman, 1997; Nicolas *et al.*, 2005, Zhang *et al.*, 2008). The extremely high number of infections and deaths as well as insufficient impact of polysaccharide vaccines, have led to initiate in 2001 the “Meningitis Vaccine

Table I
Epidemiological events with ST-5, ST-41/44, ST-32, ST-11 clonal complexes and their influence on vaccines development

Clonal complex	The most common serogroup/phenotype	Selected epidemiological occurrences	Time period	Vaccine used/developed	References
ST-5CC	Serogroup A	3 pandemics, all started in China and spread: 1. to Russia, Scandinavia and Brazil; 2. to Nepal, India, European countries, the USA and Africa; 3. to Mongolia and Moscow yearly epidemics during dry seasons and cyclical wide epidemics every 5–10 years with incidence rate reaching 1000/100 000	60s 80s 90s Since 80s	polysaccharide vaccines used in massive immunization campaigns since 2010 conjugate vaccine, MenAfriVac® in national immunization programmes	Achtman, 1997, Caugant and Nicolas, 2007, Nicolas <i>et al.</i> , 2005 www.meningvax.org
ST-41/44CC	B:4:P1.7–2,4	hyperendemic situation in New Zealand	90s–2000s	OMV vaccine MeNZB®	Holst <i>et al.</i> , 2013 Martin and McDowell, 2004
ST-32CC	B:15:P1.7,16	hyperendemic situation/prolonged outbreaks in • Norway • Oregon, the USA	60s and 70s 1990–2000s	OMV vaccine MenBvac®	Harrison <i>et al.</i> , 2009 Holst <i>et al.</i> , 2009
	B:14:P1.7,16	• Normandy, France	2000s		
	B:4:P19,15	hyperendemic situation/prolonged outbreaks in • Spain • Cuba, Brazil and South Africa	70s 80s	OMV vaccine VA-MENGO-BC®	Pannato <i>et al.</i> , 2013 Racloz and Luis, 2010
	B:15:P1.3	outbreaks in Chile	80s	OMV vaccine WRAIRc®	
ST-11CC	Serogroup C	outbreaks and increase of IMD incidence in Canada, the USA, Israel, Czech Republic, Iceland, Finland, Norway, the UK, Greece, Spain and Australia. further widespread all over the world	Since mid-80s	initially polysaccharide vaccines used in massive immunization campaigns conjugate vaccines against MenC (MCC) in national immunization programmes in many European countries and Americas	Campbell <i>et al.</i> , 2009 Halperin <i>et al.</i> , 2012 Harrison <i>et al.</i> , 2010 Jafri <i>et al.</i> , 2013 Miller <i>et al.</i> , 2002
	Serogroup W	international epidemics associated with the Hajj Pilgrimage to Mecca, when epidemic clone was transferred into 16 countries <i>i.e.</i> the USA, the UK, Nordic countries, Europe wave of epidemics in Burkina Faso with app. 12 000 cases	2000, 2001 2002	polysaccharide vaccines used in massive immunization campaigns	Hahné <i>et al.</i> , 2002, Harrison <i>et al.</i> , 2009 Mayer <i>et al.</i> , 2002, Taha <i>et al.</i> , 2000

Project”, which resulted in developing an accessible, inexpensive conjugate polysaccharide vaccine against serogroup A for Africa, MenAfriVac® (www.meningvax.org). The vaccine was introduced in December 2010, first in Burkina Faso (Djingarey *et al.*, 2012) and in accordance with the last data more than 217 million people in 15 countries have been vaccinated so far. The effects of mass vaccinations achieved so far are very promising. Decreased number of cases associated with serogroup A as well as sharp decline of the total incidence of IMD has been noticed (Daugla *et al.*, 2014; Novak *et al.*, 2012; www.meningvax.org) and MenA has disappeared from the “vaccinated” countries of the “meningitis belt” (Table I) (Caesar *et al.*, 2013).

ST-41/44CC and ST-32CC

Nowadays ST-41/44CC is the most common CC among MenB in Europe representing 31.7%. Its members are observed not only among MenB but also MenC and non-groupable meningococci (Whittaker, 2013). For example in Poland in the period 2009–2011 this CC was the third and the second most common CC among meningococci of serogroup B and C, respectively (Skoczyńska *et al.*, 2013). According to the MLST database, ST-41/44CC is very diverse, containing the highest number of STs among hypervirulent clonal complexes. At the time of writing as many as 1800 sequence types have been assigned to this CC

while ST-5CC, ST-32CC or ST-11CC include 53, 597 and 359 STs, respectively (<http://pubmlst.org/neisseria/>). At present, members of this complex are circulating worldwide, being responsible mainly for sporadic cases, but in the past it was different. For example in New Zealand, a clone of ST-41/44CC with phenotype B:4:P7-2,4 caused a threatening hyperendemic situation (Watkins and Maiden, 2012). A yearly increase in the number of IMD cases (from 53 in 1990 to 650 in 2001) forced the development of an OMV vaccine containing protein antigens of epidemic clone in order to stop its further expansion (Martin and McDowell, 2004). The vaccine proved to be effective, decreasing IMD incidence from 24.7/100 000 in 2001 to 2.6/100 000 in 2008 (Table I) (Holst *et al.*, 2013). Taking this success into consideration as well as the clone with phenotype B:P7-2,4 worldwide distribution and OMVs adjuvant properties, vesicles from MenZB[®] vaccine have been used recently in a new 4-component vaccine 4CMenB, Bexsero[®]. The vaccine contains also other protein antigens like factor H-binding protein (fHbp), neisserial heparin-binding antigen (NHBA) and neisserial adhesin A (NadA) identified by reverse vaccinology (Caesar *et al.*, 2013; Pannato *et al.*, 2013; Serruto *et al.*, 2012). Analysis of MenB conducted in many countries showed high predicted coverage by the 4CMenB vaccine and it is believed that this vaccine will be efficacious against the majority of serogroup B meningococci (Vogel *et al.*, 2013).

Second, the most common CC among MenB is ST-32CC. In 2011 meningococci B/ST-32CC represented 27.8% of MenB responsible for IMD in Europe while in Poland between 2009–2011 ST-32CC meningococci were predominant and represented 32.4% of MenB (Skoczyńska *et al.*, 2013; Whittaker, 2013). ST-32CC isolates have been related mainly to sporadic IMD. However, in the past, as already mentioned Men-41/44CC, meningococci of this complex were the cause of few prolonged epidemics. The first report of epidemic potential of ST-32CC came from Norway, from 1969. Then its spread throughout European countries, South Africa and the Americas was observed (Harrison *et al.*, 2009; Harrison *et al.*, 2010; Racloz and Luiz, 2010). According to the MLST database and phenotyping analysis, three epidemic clones were characterized. To control these strain-specific serogroup B epidemics, three OMV vaccines were developed (Table I). Although their safety and effectiveness was proven and a significant decrease in the incidence of IMD was observed, for years they were used for protection against strains that were used to construct the vaccines only. Later on, their utility against strains sharing some antigens with vaccine strain was proven, as was shown in Normandy (Caron *et al.*, 2011; Holst *et al.*, 2009; Pannato *et al.*, 2013).

ST-11CC

Meningococci belonging to ST-11CC have had a significant impact on global meningococcal epidemiology and advances in strategies to control and prevent IMD. In contrast to other clonal complexes, ST-11CC isolates have been present among meningococci of several serogroups including B, C, W and Y (<http://pubmlst.org/neisseria/>, Barroso *et al.*, 2013), although the majority represent MenC. In Europe in 2011 60% of MenC belonged to ST-11CC whereas in Poland in the period 2009–2011 only 11.3% (Skoczyńska *et al.*, 2013; Whittaker, 2013). They are well known for their unique epidemic potential. In the mid-1980s and 1990s several local outbreaks as well as an increase in IMD incidence caused by MenC/ST-11CC occurred in some provinces of Canada. It is threatening that at the same time ST-11 widely spread into other countries on all continents (Table I) (Ashton *et al.*, 1991; Gottfredsson *et al.*, 2006; Harrison *et al.*, 2010; Jelfs *et al.*, 2000; Kremastinou *et al.*, 1999; Krizova and Musilek, 1995; Miller *et al.*, 2001; Tribe *et al.*, 2002). During the following years a next wave of IMD caused by ST-11 clone in Canada was observed (Tsang *et al.*, 2004; Zhou *et al.*, 2012) and isolates belonging to this CC have become predominant among MenC all over the world causing more numerous outbreaks and clusters *i.e.* in educational institutions, military barracks, or other crowded, semi-closed places. (Bijlsma *et al.*, 2014; Brehony *et al.*, 2007; Chacon-Cruz *et al.*, 2014; Deghmane *et al.*, 2010; de Lemos *et al.*, 2007; ECDC, 2013; Fazio *et al.*, 2009; Garnier *et al.*, 2011; Simon *et al.*, 2013). Also in Poland between 2006–2009 several outbreaks were recorded as well as an increase of incidence of MenC ST-11CC (Fig. 2) (Grecki and Bienias, 2006; Kadłubowski *et al.*, 2007; Skoczynska *et al.*, 2010; Waśko *et al.*, 2009).

It is necessary to emphasize that infections caused by ST-11CC meningococci of serogroup C are associated with significantly higher CFR and sequelae rates in comparison to isolates of other CCs. This can be due to the fact that ST-11CC isolates more frequently cause septicemia than meningitis and mortality from septicemia is generally higher (Rosenstein *et al.*, 2001). It was also interesting that outbreaks caused by the mentioned clone affected mainly teenagers and young adults (Ashton *et al.*, 1991; Jensen *et al.*, 2003; Krizova and Musilek, 1995; Skoczynska *et al.*, 2013; Smith *et al.*, 2006; Trotter *et al.*, 2002). Because of frequent outbreaks occurrence, significant increase in incidence and higher mortality, massive immunization campaigns were carried out. Initially, available polysaccharide vaccines were widely used, for example in Canada and Czech Republic (De Wals *et al.*, 1996; Kriz *et al.*, 1995). Alarming changes in epidemiology accelerated the development of conjugate vaccines against



Fig. 2. Map of Poland showing regions where seven outbreaks (I–VII) caused by ST-11CC isolates between 2006 and 2009 took place (in the brackets is the number of reported cases).

MenC (MCC), rapidly introduced for the first time in 1999 in the UK. It is interesting and unique that their introduction was initiated without any evidence of the vaccines' efficacy (it was extrapolated from polysaccharide vaccines), only with safety and immunogenicity confirmation (Miller *et al.*, 2001). MCC vaccines apart from dramatic decrease of the incidence of MenC IMD in vaccinees, reduced also nasopharyngeal carriage, resulted in desirable herd immunity effect (Campbell *et al.*, 2009; Larrauri *et al.*, 2005; Miller *et al.*, 2001). Consequently, the vaccine was successively introduced into national immunization programmes in some other European countries as well as in Australia, Canada and South America (Halperin *et al.*, 2012; Jafri *et al.*, 2013).

Although ST-11 clone of serogroup C was the cause of many outbreaks and epidemics all over the world, the largest epidemics were caused by another variant of ST-11 meningococci, possessing W polysaccharide capsule. The first two international epidemics took place in 2000 and 2001, and were associated with the Hajj Pilgrimage to Mecca. Asymptomatic carrier pilgrims returned to the countries of their origin and transferred epidemic clone to their relatives/contacts

in few continents (Hahné *et al.*, 2002; Mayer *et al.*, 2002; Taha *et al.*, 2000). Later, in 2002, a wave of epidemics was also reported in Burkina Faso (Table I) (Harrison *et al.*, 2009). Currently changes in the distribution of W/ST-11 clone have been observed *e.g.* after the introduction of a conjugate vaccine against MenA in some African countries like Burkina Faso, where the clone has become predominant (Hossain *et al.*, 2013; MacNeil *et al.*, 2014). Spread and/or a noticeable increase of cases caused by the clone has been noted recently also in Europe, South America and Asia (Barra *et al.*, 2013; Barroso *et al.*, 2013; Ladhani *et al.*, 2014; Yamamoto *et al.*, 2013; Zhou *et al.*, 2013).

Concluding remarks. It is still unknown why some meningococcal clones may persist for decades and are characterized by unusual high virulence or epidemic potential, including sudden, drastic increase of incidence, hyperendemic IMD, and outbreaks/epidemics, whereas others are quickly replaced and disappear. As a consequence, the epidemiological situation of IMD is dynamic and variable worldwide and greatly depends on the emergence and widespread of clones belong-

ing to hyperinvasive clonal complexes as e.g. ST-5CC, ST-11CC, ST-32CC and ST-41/44CC. Their occurrence has serious implications for health policy, often requiring mass immunization campaigns. Paradoxically, alarming situations caused by the mentioned CCs have stimulated the development and introduction of new vaccines, counting OMV vaccines against few epidemic clones, different conjugates against serogroups A, C, W, Y meningococci and recent protein vaccines targeting mostly MenB. Experience gained during mass vaccinations has confirmed that immunoprophylaxis is the best and the most effective means to control IMD. Despite the unquestionable success of vaccination, isolates of hyperinvasive clones constitute a continuous public health threat, because they are constantly circulating and able to modify their antigenic profiles to escape host immune response. Therefore, continuous monitoring of meningococcal isolates including thorough molecular typing is indispensable and fundamental for taking appropriate preventive measures.

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Prevalence of Polyoma BK Virus (BKPyV), Epstein-Barr Virus (EBV) and Human Papilloma Virus (HPV) in Oropharyngeal Cancer

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Abstract

The aim of this study was to analyze the prevalence of BK virus, Human Papillomavirus and Epstein-Barr virus in oropharyngeal cancer, and to test our hypothesis that BKV/HPV/EBV co-infection plays a role in oropharyngeal squamous cell carcinoma. The correlation between viral infection, OSCC, anatomic location, pre-treatment staging, evidence of metastases to lymph nodes, and grading was also investigated. The examination samples were collected from 62 patients from paraffin tissue blocks. Males (90.3%) with, smoking (83.9%) and alcohol abuse (67.7%) problems prevailed in the studied group. G2 histological type was recognized in 80.6% cases. T4 (77.4%) and N2 (56.5%) traits occurred in the majority of patients. No cases of metastasis were observed (M0 100%). HPV – 24.2%, EBV – 27.4% and BKV 17.7% were detected in the studied samples. We observed co-infection EBV/BKV in 8% of cases, HPV/BKV in 4.8%, and HPV/EBV in 9% cases. Only in two cases co-infection of all three viruses was found.

Key words: BKPyV, Epstein-Barr virus (EBV), Human Papilloma virus (HPV), oral squamous cell carcinoma (OSCC), oropharynx

Introduction

Head and neck cancer is a very important problem in public health worldwide and as well as in Poland. In Poland oral and oropharyngeal cancer constitutes 3.8% cancers among men and 1.3% cancers among women (Didkowska *et al.*, 2013). Squamous cell carcinoma (SCC) constitutes 90% of all cases of cancers localised in the oral cavity and the oropharynx. The etiology of OSCC is considered to be multifactorial. Oncogenic viruses have also been involved in OSCC development (Hillbertz *et al.*, 2012; Scully, 2011; Alibek *et al.*, 2013). The cancerogenicity of HPV in humans was conducted by the International Agency for Research on Cancer (IARC) in 2007 and 2012. It was concluded that there is evidence indicating that HPV 16 can cause oral cancer (IARC, 2007; IARC, 2012). Ten to thirty percent of oral cancers are associated with HPV.

A number of studies also suggest the participation of Epstein-Barr virus (EBV) in oral cancer (Acharya *et al.*, 2014; Kis *et al.*, 2009; Jalouli *et al.*, 2010; Jalouli *et al.*, 2012). It is one of the most common viruses in humans (Alipov *et al.*, 2005). EBV was the first human virus to which oncogenic potential was attributed, and it is

classified as group 1 carcinogen by the World Health Organization's International Agency for Research on Cancer (IARC, 1997). EBV is correlated with nasopharyngeal and gastric carcinoma, squamous cell carcinoma, Hodgkin's lymphoma and Burkitt's lymphoma (Peh *et al.*, 2003; Slots, 2005; Slots *et al.*, 2006; Tiwawech *et al.*, 2008; Perera *et al.*, 2010; Chen, 2011).

The BK polyomavirus (BKPyV) is a small, non-enveloped, circular double-stranded DNA virus, belonging to the *Polyomaviridae* family, genus *Orthopolyomavirus*. The *Orthopolyomavirus* genus includes the JC virus (JCPyV), BK virus (BKPyV), the Simian virus SV40, Merkel cell polyomavirus (MCPyV) and trichodysplasia spinulosa-associated polyomavirus (Benett *et al.*, 2012). All polyomaviruses encode regulatory proteins, named large and small tumor antigens (LT-ag and ST-ag). The LT-ag has oncogenic potential in cell culture and animal models. It is estimated that up to 90% of the general population may be BKV infected, mainly during childhood (between 5 and 10 years of age). The transmission route is not clearly defined; however, the orofecal and respiratory route transmission is most possible as the upper respiratory tissues are more susceptible to infections. Additionally, BKV-induced

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nephropathy is a well-known problem among kidney transplant recipients. What is more, the BKV DNA was detected in human brain tumors, in neuroblastoma, in urinary tract tumors, in carcinomas of the uterine cervix, vulva, lips and tongue, and in Kaposi's sarcoma. (Tognon *et al.*, 2003; Neiryneck *et al.*, 2012; Schowalter *et al.*, 2012; Raeesi *et al.*, 2012; Konietzny *et al.*, 2012). The correlation between BKV and metastatic bladder carcinoma among immunosuppressed transplant recipients, and among BKV and prostate and bladder carcinoma was analyzed. It is probably consequence of the kidneys being the main site of BKV latency (Tognon *et al.*, 2003). On the other hand, salivary glands are described as a potential location of the virus as the presence of BKV genetic material was detected in saliva (Jeffers *et al.*, 2009). The BKV virus is detected in the oral cavity, and probably has tropism to squamous cells.

The aim of this study was to analyze the prevalence of BK virus, Human Papillomavirus and Epstein-Barr virus in oropharyngeal cancer, and to test our hypothesis that BKV/HPV/EBV co-infection plays a role in oropharyngeal squamous cell carcinoma.

Experimental

Materials and Methods

The present study comprised a group of 62 patients with a diagnosed and histopathologically confirmed OSCC who were hospitalized between 1995–2005 at the Chair and Department of Otolaryngology and Laryngological Oncology of the Medical University of Lublin, Poland. The patients had not received radiotherapy or chemotherapy before. TNM classification was done according to the criteria of the Union Against Cancer (UICC) (Sobin *et al.*, 2009). Histological grading was performed according to the World Health Organization criteria, which divide tumors into three types: well differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3) (Cardesa *et al.*, 2010).

DNA extraction. DNA was extracted from three, 10- μ m thick sections of formalin-fixed and paraffin-embedded tissue. The samples were transferred to Eppendorf tubes after cutting deep into the block. The microtome blade was changed after each time. One milliliter of xylene was added into test-tubes, mixed using puls-vortexing, and later centrifuged (3 min, 8000 rpm). Then the supernatant was removed. One milliliter of 96% ethanol was added to remove the residual xylene. The samples were vortexed and then centrifuged at 800 rpm for 3 minutes. The supernatant was carefully removed. Opened tubes were incubated at 37°C until residual ethanol evaporated.

DNA measuring. Measurement of DNA concentration was made using spectrophotometry, and β -glo-

bin gene was amplified to evaluate the DNA extraction process and the presence of amplification reaction inhibitors.

Detection of BKV. The polymerase chain reaction (PCR) method was used to detect the BK/JC virus in the specimens. With the aim of detecting the genetic material of the BK/JC virus, the primers described for the first time by Arthur *et al.* (1989) were used (Table I). The oligonucleotides attach to a highly conservative region of early coding T-Ag. Because of a high homology of BKV and JCV genomes (75%), polymers are complimentary to the DNA of both viruses. The described primer pair can therefore be used for detecting both BK and JC viruses. The PCR product sequence is specific for a given virus. Primers amplify a 176 bp fragment of BKV genetic material, and 173 bp fragment of JCV genetic material. The final concentrations of the PCR reaction mixture were as follows: 2.0 mM MgCl₂, 200 μ M dNTPs, 0.25 μ M of each primer, 0.5 U Hot Start Taq DNA polymerase (Qiagen/Hilden/Germany). Amplification was performed under the following conditions: initial denaturation 94°C 15 min., followed by 40 cycles: 94°C 1 min., 55°C 1 min., 72°C 1 min.; final extension: 72°C 10 min. During each PCR

Table I
Epidemiological and clinical characteristics of patients.

		N = 62	%
sex	female	6	9.7
	male	56	90.3
age	40–49	12	19.4
	50–59	46	74.2
	60 ≤	4	6.4
place of residence	urban	37	59.7
	rural	25	40.3
smoking	yes	52	83.9
	no/no answer	10	16.1
alcohol abuse	yes	42	67.7
	no/no answer	20	32.3
histology stage G	1	2	3.2
	2	50	80.6
	3	10	16.2
T stage	1	–	–
	2	2	3.2
	3	12	19.4
	4	48	77.4
N stage	No	11	17.7
	N1	14	22.6
	N2	35	56.5
	N3	2	3.2
M stage	0	62	100.0
	1	0	0

Table II
The primer sequences used for the studied viruses.

Virus	Primer sequences	Product size
BKPyV	PEP-1 (5'-AGTCTTTAGGGTCTTCTACC-3') PEP-2 (5'-GGTGCCAACCTATGGAACAG-3')	176 bp
Epstein-Barr virus	Outer 5'-GTC ATC TAC GGG GAC ACG GA-3' 5'-AAG AAG AGA TAT GTG GGG GT-3' Inner 5'-ACC CGG AGC CTG TTT GTG GC-3' 5'-GGA GAA GGT CTT CTC GGC CTC-3'	54 bp

run, the samples were tested, together with one negative and one positive control. DNA from urine of a kidney transplant patient was used as a positive PCR control to assess the success of amplification (ATCC VR-837). PCR reagents without template DNA served as a negative control. The PCR products were analyzed using electrophoresis in 2% agarose gel.

Detection of EBV. Nested PCR was carried out for detection of EBV DNA (54 bp). The primer sequences are shown in Table II.

All PCR reactions were carried out in the final volume of 25 µl using Taq PCR Core Kit (Qiagen/Hilden/Germany). The concentrations of PCR reaction components were prepared as follows: 2 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of each forward and reverse primer and 0.5 U of Taq DNA polymerase. During each run, the samples were analyzed together with one negative and positive control. The negative control consisted of nuclease-free water, while the positive control for EBV was EBV-positive cell line, Namalwa, ATCC-CRL-1432. The reaction mixture containing 5 µl of extracted DNA was amplified under the following conditions: 94°C for 3 minutes of initial denaturation, then 35 cycles of 94°C for 30 seconds, 55°C for 40 seconds, 72°C for 1 minute with the final extension at 72°C for 5 minutes. The second round amplification was performed with 1 µl of the first run product in the same conditions. The final PCR products were analyzed on 3% agarose gel.

HPV detection and genotyping. HPV detection and genotyping was performed using the INNO-LiPA HPV Genotyping Extraassay (Innogenetics/Gent/Belgium). The kit is based on the amplification of a 65 bp fragment from the L1 region of the HPV genome with SPF10 primer set. PCR products are subsequently typed with the reverse hybridization assay. PCR products are subsequently typed with the reverse hybridization assay. This kit identifies 28 HPV genotypes: HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 52, 52, 53, 54, 56, 58, 59, 66, 68, 69, 70, 71, 73, 74, and 82.

Statistical analysis. Statistical analysis was performed to investigate the relationship between EBV and HPV presence and clinical and demographical

characteristics of patients were determined by means of Pearson's chi-square test and with Kruskal-Wallis test for small groups. Statistical significance was defined as $p < 0.05$.

This research was approved by the Ethics Committee and is in accordance with the GCP regulations (no. KE-0254/133/2013).

Results

Males (90.3%) with, smoking (83.9%) and alcohol abuse (67.7%) problems prevailed in the studied group. G2 histological type was recognized in 80.6% of cases. T4 (77.4%) and N2 (56.5%) traits occurred in the majority of patients. No cases of metastasis were observed (M0 100%). Characteristics of patients with oropharyngeal carcinoma are shown in Table I.

In the studied samples HPV was detected in 27.4%, EBV in 29%, and BKV in 17.7%. We observed co-infection EBV/BKV in 8% of cases, HPV/BKV in 4.8%, and HPV/EBV in 9.7%. Only in two cases co-infection all three viruses was detected. The presence of JCPyV was observed in none of the studied samples. There isn't statistical significance between HPV, EBV, BKV infection and sex, age, place of residence, tobacco smoking, alcohol use, G, T, N features. Detailed results are shown in Table III.

Discussion

Literature on the role of HPV virus, and especially HPV 16 in oral squamous cell carcinoma is abundant (Syrjanen *et al.*, 1983; Szostek *et al.*, 2009; Jalouli *et al.*, 2010; Jalouli *et al.*, 2012; Syrjanen and Syrjanen, 2013; Metgud *et al.*, 2014; Sand and Jalouli, 2014; Sathish *et al.*, 2014). The frequency of HPV occurrence varies in different populations and is dependent on the kind of the clinical material and the applied research methods. According to the available data, the prevalence of HPV in squamous cell carcinoma of the oral cavity

Table III
Correlation between HPV, EBV, BKV and epidemiological and histological grades and TN classification.

		HPV + (N=17)			EBV + (N=18)			BKV + (N=11)		
		n	%	p	n	%	p	n	%	p
sex	female	2	88.2	0.6234	15	83.3	0.2338	10	90.9	0.9422
	male	15	11.8		3	16.7		1	9.1	
age	40–49	6	35.3	0.0698	1	5.6	0.1954	3	27.3	0.6775
	50–59	10	58.8		16	88.8		7	63.6	
	60 ≤	1	5.9		1	5.6		1	9.1	
place of residence	urban	9	52.9	0.4725	12	66.7	0.0595	7	63.6	0.7679
	rural	8	47.1		6	33.3		4	36.4	
smoking	yes	13	76.5	0.7962	13	72.2	0.0595	10	90.9	0.1527
	no/no answer	4	23.5		5	27.8		1	9.1	
alcohol	yes	11	64.7	0.7860	11	61.1	0.6593	9	81.8	0.1527
	no/no answer	6	35.3		7	38.9		2	18.2	
G	G1	0	0	0.7838	1	5.6	0.7838	1	9.1	0.3987
	G2	15	88.2		13	72.2		9	81.8	
	G3	2	11.8		4	22.2		1	9.1	
T	T2	2	11.8	0.4855	0	0	0.1527	10	90.9	0.4786
	T3	0	0		6	33.3		1	9.1	
	T4	15	88.2		12	66.7		0	0	
N	N0	5	29.4	0.3939	3	16.7	0.3144	3	27.3	0.1649
	N1	4	23.5		4	22.2		1	9.1	
	N2	8	47.1		11	61.1		7	63.6	
	N3	0	0		0	0		0	0	

and oropharynx varies and ranges from 8% to 74% (Castro and Busolotti, 2006; Ritchie *et al.*, 2003; Sathish *et al.*, 2014). In the present study, HPV DNA was detected in formalin-fixed and paraffin-embedded tissue in 24.2% cases. In our earlier studies from fresh frozen tissue, HPV was detected in 32.5% (HPV 16 composed 22.5%) (Polz-Gruszka *et al.*, 2015). The presence of EBV was found in 27.4% of the studied patients. The role of EBV virus in OSCC was first observed by zur Hausen (1976), while in Poland by Szkaradkiewicz *et al.* (2002). Other authors also emphasize the role of EBV in the development of OSCC (Kis *et al.*, 2009; Jaloluli *et al.*, 2010; Jaloluli *et al.*, 2011; Senyuta *et al.*, 2013). Jaloluli *et al.* (2012) detected the presence of EBV in 55% of samples from 8 different countries. A number of studies point to the co-infection by HPV and EBV in cervix cancer (Szkaradkiewicz *et al.*, 2004) and in oral squamous cell carcinoma (Al Mustafa *et al.*, 2009; Achayra *et al.*, 2014; Jiang *et al.*, 2015). Several articles indicate that co-infection by multiple oncogenic viruses may be an important risk factor in the development of OSCC (Jalouli *et al.*, 2011; Achayra *et al.*, 2014; Jiang *et al.*, 2014; Sand *et al.*, 2014). Al Moustafa *et al.* (2009) showed that high-risk HPV and EBV co-infections play an important role in initiating neoplastic transformation of human oral epithelial cells.

In our study co-infection HPV/EBV was identified in 9.7%. Jiang *et al.* (2014) suggest that co-infected cells can have a higher tumorigenic potential than normal cells.

BKV DNA was also detected (17.7%) in the studied material from oropharyngeal cancer. In our earlier studies of the clinical material from oral cavity carcinoma BKV DNA was detected in 18.5% cases (Polz *et al.*, 2015). Recent data suggested a correlation between BK virus and various types of human cancers, Kaposi's sarcoma, brain tumors, and tumors of the urinary tract (Hachana *et al.*, 2012). The role of BKV in oral squamous cell carcinoma is controversial. What is more, BKV DNA presence was confirmed in high-grade squamous intraepithelial cervical lesions (precancerous lesions) (Comar *et al.*, 2011). Some authors suggest that BK virus may be a potential co-factor for HPV in the development of cervical neoplasia (Fraase *et al.*, 2011), especially together with the HPV genotype 16 (Comar *et al.*, 2011). Burger-Calderon *et al.* (2014) suggests a connection between BKPyV and the oral compartment. BKV DNA was detected in tonsillar biopsy specimens and nasopharyngeal aspirates. Besides, Moens *et al.* (2014) suggest that polyomaviruses, including those induced by other oncogenic viruses, may be a co-factor in the development of cancer. In our studies HPV/BKV co-infection was detected in 4.8% cases, while

EBV/BKV in 8%. In the light of our research results and the opinions of other researchers, we cannot exclude the role of BKV in oral SCC, taking into account the fact that the genetic material of BKV was detected in saliva (Jeffers *et al.*, 2009). Double or mixed infection with other oncogenic viruses may induce transformation. The question remains open whether BKPyV play a role in oral squamous cell carcinoma or it is a co-factor for cancers induced by other oncoviruses. It is well-known that chronic infection affects the immunological response of the host. Primary infection with a nononcogenic virus may promote superinfection with oncogenic virus capable of neoplastic transformation. The oncogenic potential of HPV is related to the expression of E6 and E7, whereas the oncogenic potential of EBV – to the expression of LMP-1 and LMP-2 and of BKV-LTag. Toll-like receptors (TLRs) play a critical role in the early innate immune response to invading pathogens by sensing microorganism and they are involved in sensing endogenous danger signals. LTag of virus BKPyV as well as the protein of LMP-1 of virus EBV lowers the expression of TLR9, which can favour the aforementioned superinfection (Moens *et al.*, 2014). According to Siennicka *et al.* (2013), TLRs simulation with microbial ligands influences EBV replication.

These observations provide insights for future studies of EBV and BKPyV pathogenesis and the association with oral squamous cell carcinoma. Future studies on the mechanisms of co-infection and/or superinfection and their role in oral squamous cell carcinoma are necessary. The knowledge about these viruses may provide targets for therapy and for diagnostic methods.

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Biodecolorization and Bioremediation of Denim Industrial Wastewater by Adapted Bacterial Consortium Immobilized on Inert Polyurethane Foam (PUF) Matrix: A First Approach with Biobarrier Model

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Abstract

The present experiments were studied on bioremediation of denim industry wastewater by using polyurethane foam (PU foam) immobilized bacterial cells. About 30 indigenous adapted bacterial strains were isolated from denim textile effluent out of which only four isolates were found to be efficient against crude indigo carmine degradation using broth decolorization method. The selected bacterial strains were identified as *Actinomyces* sp., (PK07), *Pseudomonas* sp., (PK18), *Stenotrophomonas* sp., (PK23) and *Staphylococcus* sp., (PK28) based on microscopic and biochemical characteristics. The bacterial immobilized cells have the highest number of viable cells (PK07, PK18, PK23 and PK28 appeared to be 1×10^8 , 1×10^9 , 1×10^6 and 1×10^7 CFU/ml respectively) and maximum attachment efficiency of 92% on PU foam. The complete degradation using a consortium of PU foam immobilized cells was achieved at pH 6, 27°C, 100% of substrate concentration and allowed to develop biofilm for one day (1.5% W/V). In SEM analysis, it was found that immobilization of bacterial cells using PUF stably maintained the production of various extracellular enzymes at levels higher than achieved with suspended forms. Finally, isatin and anthranilic acid were found to be degradation products by NMR and TLC. The decolorized dye was not toxic to monkey kidney cell (HBL 100) at a concentration of 50 μ l and 95% of cell viability was retained. A mathematical model that describes bacterial transport with biodegradation involves a set of coupled reaction equations with non-standard numerical approach based on the time step scheme.

Key words: biobarrier model, biofilm, bioremediation, cytotoxicity test, polyurethane foam, secondary metabolites

Introduction

The textile industry involves a wide range of raw materials, machineries and processes to engineer the required shape and properties of the final products. Indigo dyes are used by a wide number of industries and textile mills predominantly use them. Today denim is the basis of the Indian fashion industry in many cases. It is a challenging dye to use because of its insoluble nature in water; to be dissolved, it must undergo a chemical change under high alkaline condition. When a submerged fabric is removed from the dyebath, the indigo quickly combines with oxygen in the air and reverts to its insoluble form producing color on the fabric (Ciardelli *et al.*, 2001; Bes-Pia *et al.*, 2004). This textile chain begins with the production of raw fiber

continues with pretreatment, dyeing, finishing, printing, coating, and other processes. Among these processes, dyeing and finishing are major water consuming processes that generate highly polluted effluents.

The dyeing step in the textile production has the largest risk on the environment due to high concentrations of organic dyes, additives and salts used. Therefore, among the processes applied in the textile industry, dyeing process wastewater should be dealt with seriously. The districts Coimbatore, Tirupur and Karur are largely polluted (Ranganathan *et al.*, 2007) with discharged toxic wastes from dyeing and bleaching units. The problem of disposal of textile wastes is likely to become serious in the days ahead and there is possibility of affecting the groundwater in the study area. Most of the time, this process constitutes the major part

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of the water consumption and generates wastewaters distinguished by high chemical oxygen demand (COD), high dissolved and suspended solids, and high color contents (Azbar and Tonar, 2004; Chan *et al.*, 2008). Many of the conventional and even advanced treatment technologies suffer the limitation of not being able to treat highly colored wastewaters from textile manufacturing units (Vandevivere *et al.*, 1998). One of the main drawbacks of these treatments is their high energy costs and low efficiency in degrading the dye stuffs. In some studies on membrane treatment of denim textile mill effluents for reuse, the wastewater is directly filtered after a pretreatment to remove only coarse particles to reduce membrane fouling and this problem has not yet been solved (Chatcharee, 2011). An alternative method of sludge management involves use of microorganisms for in situ degradation in the soil. It has been successfully applied for cleanup of soil, surface water, groundwater, sediments and ecosystem restoration (Prasad, 2011). It has been unequivocally demonstrated that a number of xenobiotics can be cleaned up through bioremediation.

Biofilms can be composed of either single or multiple species. It provides the benefit of a stable environment for the enclosed microbes, which makes them such a prolific and widespread phenomenon in nature. The aim of the present study was the screening of potent adapted bacterial strains from the denim effluent sample. These strains were allowed to form a biofilm on an inert substrate like the polyurethane foam and retain their ability to efficiently degrade H-chromophore of Indigo dye in several optimum conditions and to check their efficiency on an industrial scale.

Experimental

Materials and Methods

Sample collection. The textile effluent sample contaminated with crude indigo dye was collected from the outlet of denim industry dyeing unit (indigo dye) in Perundurai KG Fabrics, Coimbatore. The sample was brought to laboratory and stored at 7°C and used for further process.

Isolation, screening and identification of adapted bacterial strains from denim textile effluent. The dilution plates after incubation showed zone of clearance around number of colonies. Among this only 30 predominant cultures were isolated. The zone of clearance was due to the dye degrading ability of the isolated organisms. A zone of clearance was observed after 24 hrs in the nutrient agar plate (0.5% peptone, 0.3% yeast extract, 1.5% agar and 0.5% NaCl) containing 0.1% dye. The plates were incubated at 37°C as for 24 hours. The cultures which showed a zone of clearance around their

colonies were isolated and used for further screening on nutrient broth (0.5% peptone, 0.3% yeast extract and 0.5% NaCl) (Rajendran *et al.*, 2011) amended with 0.1% of indigo carmine separately for the quantitative estimation of decolorization. The decolorization pattern was calculated by using formula *i.e.* (Initial OD-Final OD)/ Initial OD X 100. After incubation the tubes were centrifuged and the dye decolorization was measured spectrophotometrically at 610 nm. The selected bacterial strains (PK07, PK18, PK23 and PK28) were examined for their shape, gram staining and motility. The biochemical tests were performed according to Bergey's manual of systemic bacteriology (Holt *et al.*, 1994). The selected bacterial stains were identified through 16S rRNA which was carried out at Chromous Biotech Pvt Ltd, Bangalore, India.

Characterization of untreated denim industrial effluent. The raw effluent was characterized by measuring the values of 11 different physico-chemical parameters. These parameters (Total Dissolved Solids (TDS) (mg/l), Total Solids (TS) (mg/l), Chemical Oxygen Demand (COD) (mg/l), pH, color, turbidity (NTU), hardness (mg/l), conductivity (mS), resistivity (Ω) and alkalinity (mg/l)) were chosen in accordance with the regulations of Tamil Nadu Pollution Control Board. All the above mentioned physico-chemical analyses were done immediately after the effluent sample was collected (APHA, 1992).

Toxicity Test for Polyurethane foam (PUF): About 0.1 ml of 3-day old liquid nutrient broth cultures were individually spread over on nutrient agar plate (Jerabkova *et al.*, 1997). The PUF of each density was cut into slices of about 1 × 1 cm and the plates without PUF were used as a control. The plates were incubated at 37°C for 2 days. The area around and in contact with the PUF cubes were observed for bacterial growth.

Enumeration of viable cells immobilized on polyurethane foam: Polyurethane foam cubes were weighed and placed into a 100 ml of conical flask containing 20 ml of nutrient broth and autoclaved at 121°C for 15 min, 2 ml of 3 day old cultures were then inoculated separately into each flask. The cultures were incubated at 30°C with orbital shaking (120 rpm) for ten days. Enumeration of viable cells was carried out 2, 4, 6, 8 and 10 days after incubation. To ensure that only the immobilized cells were further quantified, the PUF was first rinsed with sterile nutrient broth. It was torn into pieces using sterile forceps and then suspended in nutrient broth and vortexed to dislodge the immobilized cells. About 0.1 ml aliquots were spread onto nutrient agar plate and incubated at 37°C until colonies appeared. The experiment was repeated four to five times. The attachment efficiency (AE) was calculated as the fraction of the total viable cells that was immobilized (Jerabkova *et al.*, 1997).

Comparison of treatment trails on Bioremediation of DENIM industry wastewater by pure broth culture, alginate immobilized cells and PU foam immobilized cells. Using broth cultures: To 95 ml of the effluent sample, 5 ml of individual strains and consortium (combination of four bacterial strains) were inoculated. After inoculation the samples were incubated in a metabolic shaker for 24 hours at room temperature for a period of 5 days. Samples were retrieved from the flasks after 5 days of incubation and the bioremediation efficiency of the individual cultures as well as that of the consortium were studied as described above.

Using Ca-alginate immobilized cells: The Ca-alginate entrapment of bacterial cells was performed as per the method described by Kierstan and Coughlan (1985). The three gram of beads was measured and it was added to 97 ml of denim textile effluent. This was incubated at room temperature in a metabolic shaker at 120 rpm for 5 days. Sample without inoculation was also used as a control. The treated effluent samples were retrieved and studied as described above.

Using PU foam cells: The polyurethane foam was sterilized using water at 100°C for 20 minutes and the excess water was drained off after sterilization. The pure bacterial broth cultures were added in such a way that the foam was immersed in the broth. Cultures were incubated for 24 hours at room temperature in a metabolic shaker for the biofilm to develop in the matrix of the polyurethane foam. After incubation the excess of nutrient broth was drained off and added 100 ml of denim industry wastewater. The samples were incubated in a metabolic shaker for 24 hours at room temperature for a period of 5 days. The treated effluent samples were retrieved and studied as described above.

Optimization of cultural conditions for maximum biodegradation ability (APHA, 1992). The efficient adapted bacterial consortium (four) immobilized on polyurethane foam was selected and optimized under different parameters such as retention time (on 3rd day), pH (5, 6, 7, 8 and 9), temperature (7°C, 17°C, 27°C and 37°C), Initial organic load concentration (20%, 40%, 60%, 80%, and 100%), inert substrate concentration (0.5%, 1%, 1.5%, 2% and 2.5% W/V) were used for the biofilm formation and its efficiency in biodegradation and also the decolorization.

Treatment trails of denim effluent under optimized cultural conditions: About 1.5% W/V of the foam was taken and allowed to develop biofilm for one day. A substrate concentration of 40% was added and incubated at 27°C for 3 days. The pH of the effluent was adjusted to pH 6.

Characterization of Indigo Dye Degrading Compounds Through GCMS. GC-MS is particularly useful for identification of products from disperse vat dyes. Treated and untreated effluents were centrifuged. Equal

volumes of the supernatant collected were mixed with diethyl ether separately in order to retrieve the organic content of the treated and untreated effluent samples. The organic layer was then collected and allowed to evaporate at room temperature. The residue that remains was then suspended in 5 ml 100% methanol. The methanol solution was then used for GC-MS analysis for both the treated and untreated effluent samples separately. GC-MS was performed using a THERMO GC-TRACE ULTRA VER: 5.0, THERMO MS DSQ II (version 1.10 beta, Shimadzu) (Adosinda *et al.*, 2003).

Biodecolorization and degradation of Indigo dye using various analytical methods. In order to uncover the possible mechanism of indigo dye decolorization and to identify the metabolites generated from indigo dye after bacterial treatment, various analytical techniques were used. The thin layer chromatography (TLC) (Campos *et al.*, 2001) and nuclear magnetic resonance spectroscopy (NMR) (Ramya *et al.*, 2008) have been proved to be ideal methods with the quantification of secondary metabolites from treated indigo dye. Biofilm developed on polyurethane foam was taken and air dried and SEM analysis was done. HBL 100 cells (28-homo-brassinolide/Breast cancer cell line) were purchased from National Centre for Cell Science (Pune, India) and maintained in DMEM and McCoy's medium, supplemented with non-essential amino acids. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in a CO₂ incubator. Cells were cultured and ~1 × 10⁴ cells/wells were seeded into 96 well tissue culture plates and incubated for 48 h. HBL 100 cells were treated with 50 µL concentration of processed indigo dye and crude indigo dye.

$$\text{Percentage of Cytotoxicity} = \frac{\text{Mean OD value of experimental sample}}{\text{Mean OD value of experimental control (untreated)}} \times 100$$

Note: OD – Optical Density values of treated and untreated HBL cells

Adsorption isotherm kinetics. Adsorption isotherms were studied with bacterial consortium fixed inert PUF to evaluate the dye adsorption capacity of denim industrial wastewater. The constant in the *Langmuir isotherm* was determined by plotting $C_e/(x/m)$ versus C_e and making use of Eq. 1 written as,

$$C_e/(x/m) = 1/ab + 1/a(C_e) \quad (1)$$

x/m = mass of g adsorbate (PUF), a, b = empirical constant, C_e = equilibrium concentration of adsorbate in solution after adsorption (mg/l⁻¹). In *Freundlich isotherm* was determined by plotting $C_e/(x/m)$ versus C_e and making use of Eq. 2 written as,

$$\text{Log}(x/m) = \text{Log } K_f + (1/n) \text{ log } C_e \quad (2)$$

Lagergren (Pseudo first order)

$$\text{Log}(q_e - q) = \text{Log} q_e - K_{ad} / 2.303 \times t \quad (3)$$

Whereas, q and q_e are amounts of adsorbate adsorbed (ml l^{-1}) at time, t (h) and at equilibrium respectively. K_{ad} , is the adsorption rate constant (1 per h).

Pseudo-second order

$$t/Q_t = (1/K_1 Q_e^2) + (1/Q_e) t \quad (4)$$

Where, Q_e , is the amount of adsorbate adsorbed at equilibrium (ml l^{-1}). Q_t , is the amount of adsorbate adsorbed at time t (h); K_1 , is the second order equilibrium rate constant (ml per h).

Mathematical modeling on bioremediation efficiency of multiple species used as biobarrier model.

The model of water flow, the transport of nutrients and degradation of indigo dye as well as the growth of biofilm forming bacteria are done. Considering the limiting nutrients in the simulations further nutrients are added. In this section it is discussed about qualitative results of some dual species biobarrier simulations and the multispecies biofilm experiments that could lead to the design of more effective bioremediation techniques.

In order to model multi-species biofilm interactions in porous media a three phase mixture consisting of a liquid phase, a solid PU foam phase and a biofilm phase is taken. The fundamental equation for saturated transient ground water flow of constant density in horizontal direction (E-1),

$$S_s \frac{\partial h}{\partial t} - \frac{\partial}{\partial x} \left(K \frac{\partial h}{\partial x} \right) = f \quad (E-1)$$

Statistical analysis. The statistical analysis was done by comparing various physico-chemical parameters with bioremediation of denim textile wastewater. All analysis was conducted in triplicate and results presented are the mean of triplicate \pm standard deviations (SD).

Results and Discussion

Isolation, screening and identification of adapted bacterial strains. The indigo carmine dye containing plates after incubation showed zone of clearance around number of colonies. Among these only 30 bacterial colonies which showed a zone of clearance only 4 bacterial isolates were effective in the degradation of indigo carmine by showing a reduction pattern of more than 60%. These microorganisms (PK07, PK18, PK23 and PK28) were capable of degrading the pie bond which in turn reduced the H – chromophore of the indigo carmine dye without any biosorption characters. A majority of the bacterial strains which were primarily selected

from the solid media showed biosorption characteristics in broth assay.

The UV-visible spectrophotometer showed different absorbance values for the media incubated with the 30 bacterial isolates. This variation for each culture depends on the metabolites produced by them and the ability of those metabolites to react with the dye compound and converting them into their secondary derivatives. The absorbency was measured at 610 nm (Rajendran *et al.*, 2011). From the morphological and biochemical results, all the selected adapted bacterial isolates were again identified on the basis of 16S rRNA gene sequence. Based on the sequence alignment on BLAST revealed the isolate belongs to *Actinomycetes* sp. (PK07), *Pseudomonas aeruginosa* (PK18), *Stenotrophomonas rhizophila* (PK23) and *Staphylococcus pasteurii* (PK28) with 77%, 92%, 99% and 86% sequence similarities respectively. Similar work (Balan and Monteiro, 2001) was proved among many indigo carmine degrading microorganisms and the predominant cultures.

Comparison of treatment trails on bioremediation of denim industry wastewater by pure broth culture, alginate immobilized cells and PU foam immobilized cells.

The treated effluent samples of broth cultures were checked for their bioremediation ability after 5 days of incubation. A maximum percentage of reduction was seen in consortia and their reduction percentages were COD – 61.7%, Colour – 75.38%, TS – 60.10%, TSS – 79%, TDS – 73.2%, Turbidity – 42.3%, Hardness – 63.04%, Conductivity – 14%, Resistivity – 29.3%, Salinity – 11.9%, pH – 12.2%. Mixed bacterial cultures from a wide variety of habitats have shown to decolorize the diazole linked chromophore of dye molecules in 15 days (Knapp and Newby, 1995). A specific bacterium *Pseudomonas putida* was chosen to utilize the aromatic structures.

A maximum reduction percentage was seen in bacterial consortia (Ca-alginate) and their reduction pattern were, COD – 77.3%, Colour – 78.1%, TS – 47.5%, TSS – 47.02%, TDS – 34.9%, Turbidity – 69.5%, Hardness – 54.3%, Conductivity – 5.14%, Resistivity – 45.63%, Salinity – 12.9%, pH – 11.08%. On comparing, other two treatment trails (PUF immobilized cells and free cells) were suitable for the bacterial biomass production as well as increasing the bioremediation efficiency. Therefore, it can be concluded that the entrapment technique is not suitable for the immobilization of mixed bacterial consortia because of surrounding environmental conditions (Trevors *et al.*, 1992).

A maximum percentage of reduction was seen in consortia and their reduction percentages were COD – 88.3%, Colour – 93.1%, TS – 84.5%, TSS – 50.8%, TDS – 60.9%, Turbidity – 66.7%, Hardness – 76.9%, Conductivity – 5.1%, Resistivity – 29.3%, Salinity – 11.9%, pH – 12.2%. The storage stability and microbial activity of

encapsulated cells in PUF are better than those of cells encapsulated in Ca-alginate beads as well as pure broth culture. Similar works (Kim *et al.*, 2002; Forgas *et al.*, 2004) have explained the efficiency of decolorization and biodegradation enhanced by the absorption of the dye on the biomass.

Optimization of cultural conditions for maximum biodegradation ability.

Effect of retention time. A reduction was observed for the selected bacterial combination immobilized on polyurethane foam from the first day onwards till 7 days. A significant reduction was seen in third day of incubation. The reduction percentages were shown in Table I. This was similar to the work (Kim *et al.*, 2002) has explained, a decrease of COD removal efficiency by 20% was observed in fourth day due to the sloughing of biofilm in the fixed film bioreactor.

Effect of inert substrate concentration (PUF). A maximum reduction was seen in the foam quantity of 1.5%W/V. The maximum percentage of reduction for all the parameters was shown in Table IIa. Zaiat *et al.* (2000) developed a new reactor configuration, the horizontal-flow anaerobic immobilized-biomass (HAIB) reactor utilizing polyurethane foam matrices for biomass immobilization.

Effect of initial pH. A percentage of reductions of all the parameters are shown in Table IIb. The pH 7 was considered as optimum range because only in the neutral pH organisms survive better (Rump and Krist, 1992). Increase in pH affects various other parameters present in polluted water. Highly alkaline or highly acidic pH is not suitable for the indigenous organisms present in the effluent.

Effect of incubation temperature. An efficient reduction was observed at 27°C (Table IIIa). The opti-

mal temperature for bacteria ranges from 20°C to 37°C. The metabolic activities will be higher only at this range. Thus maximum degradation was seen in 27°C. In the similar experiments (Khlifi *et al.*, 2010) were conducted at different temperatures, 20°C, 25°C, 30°C, 35°C. The maximum degradation of waste water was found at 30°C.

Effect of initial substrate concentration. When different concentration of effluent samples were supplemented for the consortium, an efficient reduction was observed at 40% of the effluent sample (Table IIIb). From the results, a significant reduction occurred in diluted organic load rather than direct effluent. This was because at very low concentrations the quantity of organic content will be low. Similar results were observed in the work of Khlifi *et al.* (2010) where optimal decolorization occurred with 20% effluent at pH 5.

Effect of incubation time for biofilm development. When biofilm was allowed to develop over increased period of incubation time (1 day up to 5 days), biofilm developed on first day of incubation was found to be more efficient in the bioremediation of effluent (Table IV). When cultures were grown as biofilm the growth rate will be faster due to matrix formation and maintain a stable log phase.

Treatment trails of denim effluent under optimized cultural conditions. The maximum degradation under all the above optimized conditions were found to be TS – 41%, TSS – 75%, Turbidity – 42%, Color – 86%, COD – 89%, pH – 21%, Conductivity – 38%, TDS – 64%, Salinity – 30%, Hardness – 54% and Resistivity – 38%.

GC-MS analysis. The compounds present for the respective peaks were 1,2-benzene dicarboxylic acid, bis (2-ethylexyl) ester, Hexadecane-2-methyl, Hexadecane-2,6,10,14-tetramethyl, octacosone. The compounds

Table I
Effect of retention time on bioremediation of denim industrial effluent (Mean and Standard deviation, n=3).

	Effect of retention time (days)						
	1	2	3	4	5	6	7
Total Solids	39.45±0.92	40.51±0.54	45.10±0.60	54.06±0.73	66.47±0.72	77.52±0.65	76.40±1.20
TSS	40.89±0.50	45.75±0.45	49.84±0.58	58.30±0.85	61.41±0.89	61.26±1.10	60.21±1.01
Turbidity	17.93±0.40	21.45±0.89	22.94±0.58	26.40±0.89	31.53±0.87	29.44±0.96	29.38±1.28
Color	45.13±0.51	53.86±0.34	60.65±0.63	63.58±0.93	74.55±0.91	72.32±0.81	71.31±1.10
COD	50.58±0.65	55.42±0.88	61.86±0.40	68.45±0.88	79.41±0.69	79.35±0.91	79.17±0.77
pH	5.11±0.24	5.63±0.65	5.67±0.54	5.86±0.91	6.69±0.97	6.39±0.79	6.41±1.30
Conductivity	41.25±1.00	43.71±0.56	48.66±0.61	60.26±0.90	70.40±1.27	70.33±0.94	70.38±1.07
TDS	16.60±0.61	30.40±1.10	35.82±0.50	49.50±1.13	59.82±0.99	58.54±0.94	57.28±0.98
Salinity	15.92±0.30	18.69±0.57	24.84±0.48	34.66±0.85	43.60±1.06	42.69±0.61	42.48±1.25
Hardness	38.05±0.50	49.07±0.52	56.01±0.65	68.32±0.89	75.53±1.17	74.88±0.91	74.42±1.29
Resistivity	22.93±0.40	28.65±0.60	34.89±0.40	48.65±1.39	56.71±0.65	52.32±0.97	49.42±1.28

Note: TSS – Total Suspended Solids; COD – Chemical Oxygen Demand; TDS – Total Dissolved Solids

Table II
Effect of inert PUF concentration (a) and initial pH (b) on bioremediation of denim industrial effluent (Mean and Standard deviation, n =3).

	(a) Effect of inert PUF concentration (g/ml)									(b) Effect of initial pH								
	0.5	1	1.5	2	2.5	5	6	7	8	9	5	6	7	8	9			
Total Solids	35.38 ± 0.88	39.07 ± 0.63	55.45 ± 0.87	49.23 ± 0.90	41.55 ± 0.63	49.62 ± 0.68	64.61 ± 0.58	56.41 ± 0.98	51.18 ± 1.03	45.47 ± 0.92								
TSS	38.61 ± 0.68	45.29 ± 0.65	72.02 ± 1.47	61.49 ± 1.31	36.54 ± 1.48	45.82 ± 0.91	59.31 ± 0.97	52.34 ± 0.93	49.25 ± 0.86	46.67 ± 1.17								
Turbidity	46.13 ± 0.19	50.85 ± 0.82	64.26 ± 0.78	48.72 ± 1.45	44.60 ± 0.73	23.70 ± 0.88	40.13 ± 1.02	31.45 ± 0.93	29.85 ± 0.48	25.48 ± 1.15								
Color	42.99 ± 0.75	61.22 ± 0.94	71.25 ± 1.10	54.87 ± 0.94	48.51 ± 0.87	49.45 ± 0.88	69.15 ± 0.77	55.34 ± 0.95	57.74 ± 0.91	50.12 ± 1.04								
COD	43.01 ± 0.56	45.82 ± 0.41	74.67 ± 1.06	51.31 ± 0.89	45.27 ± 0.87	40.19 ± 1.03	66.17 ± 0.99	55.44 ± 1.21	49.65 ± 0.51	47.18 ± 0.91								
pH	5.91 ± 0.88	6.55 ± 0.62	14.56 ± 0.79	6.95 ± 0.43	8.53 ± 0.69	7.02 ± 0.81	6.61 ± 0.54	6.42 ± 0.78	6.16 ± 0.61	5.51 ± 0.69								
Conductivity	49.56 ± 0.62	61.12 ± 0.78	78.18 ± 0.87	71.25 ± 1.00	66.02 ± 0.89	60.19 ± 0.93	72.61 ± 3.41	65.99 ± 0.79	59.56 ± 0.62	55.47 ± 0.96								
TDS	36.88 ± 0.32	45.58 ± 0.57	59.41 ± 0.77	47.49 ± 0.93	40.59 ± 1.11	33.86 ± 0.98	54.76 ± 1.11	46.30 ± 0.76	47.55 ± 1.09	46.05 ± 0.71								
Salinity	33.98 ± 1.23	45.42 ± 1.48	45.45 ± 0.46	31.15 ± 1.40	30.83 ± 1.43	55.21 ± 0.90	64.28 ± 0.96	61.45 ± 0.84	60.16 ± 1.03	45.39 ± 0.84								
Hardness	35.86 ± 0.84	39.54 ± 0.64	68.42 ± 0.68	43.52 ± 0.98	31.42 ± 1.16	41.25 ± 1.00	58.03 ± 0.72	47.80 ± 0.85	48.11 ± 0.92	45.23 ± 1.07								
Resistivity	52.06 ± 0.90	54.02 ± 0.19	72.84 ± 0.65	64.34 ± 0.87	62.25 ± 1.65	60.37 ± 1.07	70.54 ± 0.38	68.40 ± 0.91	66.51 ± 0.48	59.37 ± 0.90								

Note: TSS – Total Suspended Solids; COD – Chemical Oxygen Demand; TDS – Total Dissolved Solids

Table III
Effect of temperature (a) and initial substrate concentration (b) on bioremediation of denim industrial effluent (Mean and Standard deviation, n =3).

	(a) Effect of temperature (°C)					(b) Effect of initial substrate concentration (g)				
	7	17	27	37	0.2	0.4	0.6	0.8	1	
TS	53.2 ± 0.64	59.18 ± 0.63	75 ± 0.35	68.03 ± 0.49	40.66 ± 0.54	46.95 ± 0.49	53.51 ± 0.90	65.33 ± 0.81	76.87 ± 0.46	
TSS	30.2 ± 0.64	43.82 ± 0.37	54.25 ± 0.58	45.16 ± 0.49	39.94 ± 0.57	45.05 ± 0.65	48.98 ± 0.50	57.97 ± 0.44	61.10 ± 0.63	
Turbidity	14.59 ± 1.20	25.66 ± 0.56	38.7 ± 0.64	20.77 ± 0.52	15.95 ± 0.50	18.08 ± 0.20	26.00 ± 0.75	27.88 ± 0.44	31.51 ± 0.87	
Color	43.85 ± 0.60	48.72 ± 0.56	65.91 ± 0.79	55.14 ± 0.43	40.09 ± 0.66	53.91 ± 0.34	60.23 ± 0.99	64.00 ± 0.24	76.93 ± 0.51	
COD	40.39 ± 1.10	45.16 ± 0.33	51.23 ± 1.03	40.96 ± 0.72	48.41 ± 0.50	54.85 ± 0.48	62.10 ± 0.32	68.07 ± 0.55	79.60 ± 0.64	
pH	7.03 ± 0.20	6.83 ± 0.68	7.13 ± 0.13	7.88 ± 0.35	5.08 ± 0.24	5.00 ± 0.27	4.81 ± 0.37	5.43 ± 0.34	6.50 ± 0.63	
Conductivity	54.71 ± 0.49	60.34 ± 0.91	75.04 ± 0.42	47.99 ± 0.42	39.92 ± 0.58	43.75 ± 0.56	48.47 ± 0.65	60.55 ± 0.62	69.70 ± 0.54	
TDS	49.58 ± 0.68	58.51 ± 0.92	63.96 ± 0.67	53.72 ± 0.63	36.82 ± 0.50	38.95 ± 0.78	43.17 ± 0.76	49.67 ± 0.49	59.80 ± 0.45	
Salinity	32.40 ± 0.73	40.91 ± 0.37	54.79 ± 0.68	40.10 ± 0.51	14.89 ± 0.71	18.01 ± 0.45	24.27 ± 1.04	34.57 ± 0.72	43.79 ± 0.40	
Hardness	33.88 ± 0.52	44.21 ± 0.55	50.85 ± 0.35	41.49 ± 0.99	21.68 ± 1.15	27.96 ± 0.38	34.93 ± 0.36	48.00 ± 0.46	56.83 ± 0.45	
Resistivity	65.03 ± 0.49	68.38 ± 0.87	73.78 ± 0.59	54.01 ± 0.49	40.65 ± 0.43	51.43 ± 0.63	65.13 ± 0.84	71.28 ± 0.84	75.20 ± 0.24	

Note: TSS – Total Suspended Solids; COD – Chemical Oxygen Demand; TDS – Total Dissolved Solids

Table IV
Effect of biofilm optimization on bioremediation of denim industrial effluent
(Mean and Standard deviation, n = 3).

	Effect of biofilm optimization (days)				
	1	2	3	4	5
TS	34.38 ± 0.407	22.84 ± 1.50	21.10 ± 1.39	19.42 ± 0.89	18.30 ± 0.65
TSS	23.06 ± 0.12	20.07 ± 1.88	13.53 ± 1.13	13.54 ± 0.50	15.20 ± 0.64
Turbidity	34.79 ± 0.20	28.92 ± 1.54	31.42 ± 1.09	24.43 ± 0.81	23.06 ± 0.89
Color	65.37 ± 0.23	62.17 ± 0.81	56.19 ± 0.70	51.32 ± 0.92	49.74 ± 1.39
COD	43.27 ± 0.28	25.48 ± 0.91	35.58 ± 0.89	30.46 ± 0.74	31.74 ± 1.25
pH	12.23 ± 0.27	12.49 ± 0.73	24.31 ± 0.91	19.26 ± 0.79	16.33 ± 1.30
Conductivity	14.45 ± 0.17	22.00 ± 1.39	13.33 ± 0.84	26.25 ± 0.86	24.79 ± 1.48
TDS	46.53 ± 0.33	44.54 ± 0.88	38.33 ± 0.85	29.46 ± 0.76	31.34 ± 1.36
Salinity	50.22 ± 0.08	50.48 ± 1.03	44.31 ± 0.81	37.45 ± 2.07	35.26 ± 1.01
Hardness	35.28 ± 0.24	25.58 ± 0.85	26.73 ± 1.15	25.33 ± 0.52	24.51 ± 0.64
Resistivity	49.19 ± 0.15	36.07 ± 1.48	41.43 ± 1.00	33.48 ± 0.74	32.97 ± 0.05

Note: TSS – Total Suspended Solids; COD – Chemical Oxygen Demand; TDS – Total Dissolved Solid

present in the treated sample were found to be octadecane, S-phenyl-1-butylethene-2-(monothio) carboxylate which showed peak ranges 23.14, 19.51 and 11.88 respectively which were nontoxic. Utkarsha and Jyoti (2011) reported the degradation product of the textile industry effluent by *Penicillium* was studied by GCMS analysis.

Biodecolorization and degradation of Indigo dye using various analytical methods.

¹H-NMR analysis. The ¹H-NMR spectrum of the Indigo dye showed two singlets in the downfield and aromatic proton at 7.6 δ and corresponding to NH and two doublets at 6.9 δ and 6.8 δ, which accounts for the presence of two adjacent protons on the aromatic ring. The overall spectrum clearly depicts the structure of Indigo dye. On the other hand, the Indigo dye which has undergone effective degradation was recorded by spectrum. The end product isatin exhibited three singlets at 7.5 δ, 6.9 δ and 6.8 δ which reveal the presence of the proton of SO₃H group an aromatic proton (Ramya *et al.*, 2008).

Morphological studies of immobilized bacterial cells on PUF by scanning electron microscope (SEM). The PU foam substrates used were having highly flexible and porous surface providing large surface area for immobilizing adapted bacterial cells for biofilm formation. The bacterial biofilm consisted of heterogeneous population of short and long rods, cocci and filaments. Rile *et al.* (1999) has observed the majority of F92 cells were immobilized on the outer surfaces of PUF in SEM.

Thin layer chromatography. The indigo dye is used as a unique source of carbon and nitrogen available to adapted bacterial consortium in this assay, it indicates the possibility that indigo undergoes a biodegradation

process. The degradation was demonstrated by thin layer chromatography of treated indigo dye extract, it is observed that the control dye has slight mobility and one known metabolite *i.e.* R_f = 0.2 which indicates the presence of isatin which has been detected in the extracts where indigo was present. Very few reports are available on the biodegradation products of indigo dyes. The degradation of indigo by laccases produces isatin (indole-2,3-dione), it is further degraded to anthranilic acid (2-aminobenzoic acid) and is observed through HPL analysis (Campos *et al.*, 2001).

Cytotoxicity test. The adapted mixed bacterial secondary metabolites which were released into the treated medium during biodegradation were not cytotoxic and the cells died as a result of the toxicity of indigo dye. However the decolorized indigo dye was nontoxic to the cells at a concentration of 50 μl in 48 hours while the crude indigo dye killed over 98% of the cells. It is evident from that the percent viability of HBL 100 cells was not drastically affected when the tissue was stored at 37°C up to 3 days. Labib *et al.* (2012) reported a case of fatal poisoning in a 3-year-old child after administration of indigo for therapeutic purposes (diarrhea, vomiting and fever).

Adsorption isotherms. In Langmuir plots for adsorption, the linear plots of C_e/q_e vs C_e confirm that the adsorption follows the Langmuir isotherm model. Langmuir constants, Q₀ and b were determined from the slope and intercept of the respective plots. From the Q₀ and b values it could be depicted that PU foam immobilized bacterial cells was efficient in adsorbing the dyes. The Q₀ and b values of indigo dye containing denim wastewater adsorption by PU foam immobilized cells were 20.4 and 140.74 mg/g, respectively. There was a wide difference in adsorption capacities between

bacterial biomasses studied, in particular with indigo dye adsorption. The Freundlich equation is used for heterogenous surface energies in which the energy term, Q_0 , in the Langmuir equation varies as a function of the surface coverage, q_e , strictly due to variation in the heat of adsorption.

'n' gives an indication of favorability and K_f [mg/g (mg/l-1)ⁿ], the capacity of the adsorbent. This Freundlich adsorption isotherm was applied for the adsorption of indigo dye containing denim wastewater by PU foam immobilized cells, free cells and Ca-alginate immobilized cells in agitated mode (McKay *et al.*, 1985). In the present study, the n values were found to be in the range of 5.865 and K_f values at 85.176 for adsorption of dyes studied onto PU foam immobilized cells in agitated mode. On the other hand, the Free (0.405) and Ca-alginate immobilized cells (0.625) were noted for 'n' values ranging at less than one.

Adsorption kinetics. To study the adsorption kinetics, two kinetic models were used that include Lagergren (Pseudo-first order) and Pseudo-second order models. The linearized form of Lagergren (Lagergren, 1898) and Pseudo-second order models can be studied in below. The straight line plots of $\log(q_e - q)$ vs t (time) indicates the applicability of the Lagergren equation. The K_{ad} values were calculated from the slope of the linear plots and were observed to be in the range at $\times 10^{-2}$ 1 per min at 27°C, respectively for adsorption of indigo dye by PU foam immobilized cells in agitated mode. The adsorbent rate was about 1.4×10^{-2} 1 per min (PU foam immobilized cells) at 100 mg/l⁻¹ dye concentration, respectively. Khattri and Singh (2000) reported that the temperature did not have any significant effect on the rate constant of crystal violet adsorption on neem sawdust. The K_{ad} values obtained in the present study are comparable with these observations, only PU foam immobilized cells were effective for removal of indigo dye than other treatment trails. Initially, the validity of the two models was checked by studying the kinetics under different initial dye concentrations. For Lagergren plot (free and Ca-alginate immobilized cells), correlation coefficients were found to be 0.82 to 1, but the calculated Q_e varied widely to experimental Q_e , suggesting the sufficiency of the model to fit the kinetic data for the initial concentrations examined. The reason for these differences in the Q_e values is that there is a time lag, possibly due to a boundary layer or external resistance controlling at the beginning of the sorption process. Whereas, the PU foam immobilized cells of correlation coefficient was found to be 0.99, hence from these results, we concluded that this model was highly sufficient for removal of indigo dye. The equilibrium rate constant of second order kinetics model, K_1 , was calculated from the slope of linear plots. The values of K_1 , ranged at $0.032 \text{ g}^{-1} \text{ mg}^{-1} \text{ min} \times 10^{-2}$ for the dyes stud-

ied in PU foam immobilized cells. The data obtained in the present study showed that dye adsorption by PU foam immobilized cell biomass fits well with the pseudo second order model than Lagergren model. Correlation coefficients were mostly greater than 0.99, and the lowest correlation coefficient (0.94) was better than the first order model correlation coefficients.

Mathematical modeling on bioremediation efficiency of multiple species used as biobarrier model.

A multispecies biofilm interaction in porous media was considered with a three phase mixture consisting of a liquid phase, a solid PU foam phase and a biofilm phase. The biofilm can be considered to be part of the solid phase, it is simpler to take it as a separate phase. The four bacterial species present in the porous medium develops strong biofilm forming microbes with the nutrients. The fundamental equation for saturated transient ground water flow of constant density, in horizontal direction, can be written in the form (Allen *et al.*, 1988),

$$S_s \frac{\partial h}{\partial t} - \frac{\partial}{\partial x} \left(K \frac{\partial h}{\partial x} \right) = f \quad (1.1)$$

The single fluid flow equation (1.1) arises from the mass balance law

$$S_s \frac{\partial h}{\partial t} + \frac{\partial v}{\partial x} = f \quad (1.2)$$

Substitute for the specific discharge vector v using Darcy's law

$$v = -K \frac{\partial h}{\partial x} \quad (1.3)$$

Here $h(x, t)$ denotes the hydrolic head, S_s is the specific storage, K is the saturates hydraulic conductivity and $f(x, t)$ represents source. The specific discharge vector $v(x, t)$ called Darcy velocity represents the speed of the water. We assume there are no sources for the fluid, therefore = 0 in equation (1.1). Invoking the above simplifying assumptions to equations (1.1),

$$-\frac{\partial}{\partial x} \left(K \frac{\partial h}{\partial x} \right) = 0 \quad (1.4)$$

The transport and reaction of nutrients and contaminants, and the growth of the two microbial species are governed by a system of partial differential equations. Finally we can work only with the liquid and biofilm phases,

$$\frac{\partial}{\partial t} (\phi^i \rho C) + \frac{\partial}{\partial x} (v \rho C) - \frac{\partial}{\partial x} \left(D \frac{\partial \rho T}{\partial x} \right) = r C (\rho B, \rho K, \rho C, \rho T) \quad (1.5)$$

Here ρ_i , $i = B, K, C, T$, represents the intrinsic mass density of the contaminant degrading microbes, the strong biofilm forming microbes and nutrients respec-

tively. It follows that the small initial biobarrier-forming microbial concentrations are given by,

$$\phi(\tilde{X}_B, \tilde{X}_K) = \phi_0(1 - \tilde{X}_B - \tilde{X}_K) \quad (1.6)$$

$$K(\tilde{X}_B, \tilde{X}_K) = K_0(1 - \tilde{X}_B - \tilde{X}_K)^{nk}$$

Incorporating the above simplifying assumptions into equations (1.5) and using normalized concentrations as the unknowns yields the following governing system of differential equation,

$$\frac{\partial S_C}{\partial t} + v \frac{\partial S_C}{\partial x} - \frac{\partial}{\partial x} \left(D \frac{\partial S_C}{\partial x} \right) = -\frac{1}{Y_K} \mu^K(S_C) X_K G(X_B + X_K) \quad (1.7)$$

with γ typically small. One common assumption in Monod's growth rate for bacteria is that there is only one nutrient that limits the growth. That is, there is an excess of the other nutrients. In this case the rate of substrate utilization has been usually described by Bailey and Ollis (1986).

$$\mu^j(S_i) = \mu_{\max}^j \frac{S_i}{K_{S_i}^j + S_i}$$

$j = B, K$, where S_i is the concentration of the limiting nutrient.

For the biobarrier model;

$$\mu(S_1, S_2, \dots, S_m) = \mu_{\max}^j \prod_{i=1}^m \frac{S_i}{K_{S_i}^j + S_i} \quad (1.8)$$

$j = B, K$, is used to describe the kinetics of microbial transformations of nutrient and contaminant. In System the Monod expressions are given by,

$$\mu(S_C) = \frac{\mu_{\max}^K S_C}{K_{S_C}^K + S_C} \quad (1.9)$$

Simulations. To determine the solution of an ordinary differential equation (1.4) we use a standard finite difference method to calculate h . Then we were using numerically differentiate Darcy's law (1.3) to get the velocity field v . The temporal differentiation in the microbial species equations (1.5) uses a forward Euler time integration. The nutrients and bacterial transport equations are solved using a Ghill shooting method (Kojouharov and Chen, 2000).

The first dual-species biobarrier model (1.7) with standard Monod kinetics (1.9) has been validated in with the porous media experiments done by Cunningham *et al.* (1991) for a 1×1 cm long PU foam cube with 0.70 mm, in diameter. In our first simulation, we consider initial and boundary conditions for S_C that correspond to a high nutrient concentration experiment, *i.e.* $S_C(x, 0) = S_C(0, t) = 175 \mu\text{g/ml}$. This is clearly the case, since the thresholds are smaller than the steady

state values for the nutrient. In this scenario, the steady-state biofilm forming bacterial population density was able to degrade the contaminant Indigo dye containing denim industrial wastewater.

Conclusions. Operational stability and longevity of cells encapsulated in PU foam are significantly better than free and alginate cells. Biodegradation of industrial effluents rich in organic pollutants with immobilized bacterial strains in PU foam is an extremely versatile approach that can be used in detoxification of denim waste for longer periods, provided the process is made economical and convenient for use on a large scale.

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Isolation and Characterization of *Streptomyces* with Plant Growth Promoting Potential from Mangrove Ecosystem

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Abstract

A total of 66 actinomycetes isolates were isolated from mangroves of Andhra Pradesh, India, using various enrichment techniques and pre-treatments. The samples were collected from Coringa mangrove ecosystem and pre-treated by enrichment with CaCO₃, sodium dodecyl sulphate and phenol, plated on the media supplemented with cycloheximide (50 mg/ml), nystatin (25 mg/ml) and nalidixic acid (50 mg/ml). The population count of actinomycetes fluctuated from 1.9×10^5 to 8.0×10^5 /g soil. Out of the isolated 66 actinomycetes, 8 isolates possessing plant growth promoting potential were further studied and characterized by physiological and biochemical traits and identified by 16S rRNA gene sequencing as different species of *Streptomyces* genera.

Key words: 16S rRNA, ARDRA, IAA, mangroves, PGPR, *Streptomyces*

Introduction

Mangrove is one of the most productive ecosystems and a natural renewable resource (Kathiresan, 2003; Balachandran *et al.*, 2009). These are a unique woody plant community of intertidal coasts in the tropical and subtropical zones, which are regarded as highly productive ecosystems and abode to unexplored microbial diversity including actinomycetes (Balagurunathan *et al.*, 2010). Mangroves provide a unique ecological niche to a variety of microorganisms (Agate, 1991). About 125 species of microorganisms (bacteria, fungi, algae) have been identified from mangrove environment (Kathirvel, 1996). Exploitation of less/unexplored ecosystems for actinomycetes is highly necessary for the discovery of novel bioactive metabolites (Sahoo and Dhal, 2009).

Among the different mangrove locations Andhra Pradesh of India has dense mangrove vegetation found towards coast rather than on shore land because of the dense branching network of creeks, which exist towards the coast (RSAM, 1992). There is more mangrove vegetation on tidal flats on the western side of the Krishna delta than on its eastern side. Dense mangroves are also

seen over recent sand / mud spits on the Nizampatnam bay (RSAM, 1992). Sparse mangroves are found on the eastern side of the Krishna delta.

Actinomycetes being gram-positive bacteria showing a filamentous growth like fungi. They are aerobic and group of organisms widely spread in nature, with high G+C content (60–70 mol %) and are important sources of antibiotics (> 50%) and enzymes (Edwards, 1993; Gharaibeh *et al.*, 2003; Weber *et al.*, 2003; Shantikumar *et al.*, 2006).

Screening and isolation of promising actinomycetes from mangrove ecosystem with potential antimicrobial compounds is still a thrust area of research and it is suggested that the exploration of new areas and habitats played a vital role in the search for new microbes and novel metabolites (Horinouchi, 2002). The genus *Streptomyces* produce approximately 75% of commercially and medically useful antibiotics and 60% of antibiotics used in agriculture (Sanghvi *et al.*, 2014). Seasonal variations of antagonistic actinomycetes have been determined in selected mangrove ecosystems and highest numbers of actinomycetes during monsoon have been recorded. The microbial interrelationship in mangrove sediments does exist between bacteria and

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actinomycetes, bacteria and fungi, and fungi and actinomycetes (Rathna Kala, 1995). However, across the globe, the world's mangroves are threatened. Mangrove habitats are being destroyed as rivers are dammed, their waters diverted and the intertidal zone extensively developed for agriculture or aquaculture. Previous study showed that actinomycetes isolated from Malaysian soil have the potential to inhibit the growth of plant pathogens (Jeffrey *et al.*, 2007). Likewise actinomycetes isolated from Turkey's farming soil have the ability to inhibit *Erwinia amylovora* a bacteria that cause fireblight to apple and *Agrobacterium tumefaciens* a causal agent of Crown Gall disease (Oskay *et al.*, 2004). Besides acting agents for control of plant pathogens they also possess the capability for plant growth promotion (Nassar *et al.*, 2003; da Silva Sousa *et al.*, 2008). This is due to their capacity to produce IAA, antibiotics, siderophores, enzymes that have antimicrobial activity, substances that promote plant growth, solubilization of phosphates and competition with plant pathogens for substratum and nutrients (Hamdali *et al.*, 2008; da Silva Sousa *et al.*, 2008).

The present study aims at isolation of actinomycetes from mangrove ecosystem of Andhra Pradesh, India and to evaluate their plant growth promoting potential by siderophore and IAA production assay. The study also focusses on the genetic diversity study of the plant growth promoting isolates on the basis of morpho-physiological, biochemical and molecular characteristics.

Experimental

Materials and Methods

Soil sampling. A total of 10 soil samples and one water sample were collected from the Mangrove ecosystem of Kakinada District, Coringa (latitude 16°44'

to 16°53'N and longitude 82°14' to 82°22'E), Andhra Pradesh, India by systematic sampling method. Samples were collected from 15 cm depth and transported to the laboratory in sterile bags and air-dried at room temperature. The geographical locations, as well as physico-chemical properties of samples were recorded (Table I).

Isolation of actinomycetes. Samples were subjected to various enrichment techniques like CaCO₃ (Tsao *et al.*, 1960), SDS (Hayakawa and Nonomura, 1989), phenol (Hayakawa *et al.*, 2004), and media such as, Starch Casein Agar (SCA), Actinomycetes Isolation Agar (AIA) and Soil Extract Agar (SEA), amended with antibiotics nystatin (25 mg/ml), cycloheximide (50 mg/ml) and nalidixic acid (50 mg/ml), were employed for the isolation of *Streptomycetes*. The air dried samples were incubated at 55°C for 5 min in an incubator. One gram of soil was dissolved in 100 ml of distilled water (10⁻² dilution) and 1 ml of 10⁻³, 10⁻⁴, 10⁻⁵ serial dilutions were spread plated on media amended with antibiotics using dilution-plate technique and incubated at 30°C for 2–3 weeks. After incubation, actinomycetes colonies were selected and maintained by sub-culturing on ISP-2 agar (g/l: 4.0 g glucose; 4.0 g yeast extract; 10.0 g malt extract; 18.0 g agar) slants and stored at 4°C for further use.

Identification by polyphasic taxonomy. Out of the isolated actinomycetes eight isolates (AM2-2, AM2-3, AM2-4, AM2-7, AM2-8, AM2-10, AM2-11, AM5-16) were characterized by morpho-physiological, biochemical and molecular methods. Identification of actinomycetes was carried out, using standard methods of morphological and physiological traits (Shirling and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology (Williams *et al.*, 1989). Morphological methods consisted of macroscopic and microscopic studies. For chemotaxonomic studies, the colonies were grown in glucose yeast extract malt extract broth for 4–5 days and then filtered out and processed for determination

Table I
Details of sources of samples, pH and geographical Co-ordinates

Sample type	Source	Geographical Co-ordinates	pH	Cfu/g soil
Mangroves water sample	Nearby sea shore.	16°30"-17°N, 82°10"-80°23"E	7.4	8.0 × 10 ⁵
Rhizospheric soil	<i>Avicennia marina</i> (mangrove plant)	16° 56" N, 82° 13" E	7.2	2.25 × 10 ⁵
Rhizospheric soil	<i>Avicennia officinalis</i> (mangrove plant)	16° 56" N, 82° 13" E	7.3	1.9 × 10 ⁵
Rhizospheric soil	<i>Excoecaria agallocha</i> (mangrove plant)	16° 56" N, 82° 13" E	7.6	2.4 × 10 ⁵
Rhizospheric soil	<i>Sonneratia apetala</i> (mangrove plant)	16° 56" N, 82° 13" E	7.2	3.06 × 10 ⁵
Rhizospheric soil	<i>Aegicerale corniculatum</i> (mangrove plant)	16° 56" N, 82° 13" E	7.4	3.9 × 10 ⁵
Rhizospheric soil	Grasses growing in mangrove	16° 56" N, 82° 13" E	7.0	2.86 × 10 ⁵
Rhizospheric soil	<i>Cerriops decandra</i> (mangrove plant)	16° 56" N, 82° 13" E	7.2	3.93 × 10 ⁵
Rhizospheric soil	<i>Rhizophora conjugata</i> (mangrove plant)	16° 56" N, 82° 13" E	7.5	3.61 × 10 ⁵
Rhizospheric soil	<i>Hibiscus tetraceus</i>	16° 56" N, 82° 13" E	7.1	4.05 × 10 ⁵
Soil sample	Sample near the start of Coringa wild life sanctuary	16° 56" N, 82° 13" E	7.3	3.1 × 10 ⁵

of the diaminopimelic acids (LL-DAP or meso-DAP) isomers and whole cell sugar patterns (Lechevalier and Lechevalier, 1980) using thin layer chromatography (TLC) on precoated cellulose plates (Merck, India).

Scanning electron microscopy. Spore surface ornamentation was evaluated by Scanning electron microscopy (SEM). Mycelia were taken (after 10 days of incubation) and washed in 0.1 M sodium cacodylate buffer (pH 7.4). They were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4°C followed by post-fixation with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) and dried in a critical point dryer (EMITECH model K850, Hitachi). The preparations were mounted onto aluminium holders, sputter-coated with 10 nm Au and observed by SEM (Hitachi model S3400 at 15–30 kV, 2–5.00 µm).

Physiological characterization, plant growth promoting (PGP) attributes and extracellular enzymes production. Physiological characterization such as the effect of pH (6–9), temperature (20–45°C), salinity and carbon source utilization were studied. Carbohydrate utilization was determined by growth on carbon utilization medium (ISP-2) (Pridham and Gottlieb, 1948) supplemented with 1% carbon source (Glucose, Arabinose, Rhamnose, Mannitol, Dulcitol, Raffinose, Fructose, Sucrose, Lactose, Inositol). The intrinsic resistance of actinomycetes to salinity was evaluated according to Yadav *et al.* (2009), by observing the growth at $28 \pm 2^\circ\text{C}$ in tryptose soya broth amended with different concentrations of NaCl (2, 4, 6, 8% w/v). For growth promoting attributes, phosphate solubilization (Mehta and Nautiyal, 2001), siderophore production (You *et al.*, 2005), nitrate reduction (Glass *et al.*, 1997), cyanogenesis (Schippers *et al.*, 1990) and IAA production (Bano and Musarrat, 2003) were evaluated. The extracellular enzyme activity was assayed using standard methods such as amylase (Mishra and Behera, 2008), protease (Manachini *et al.*, 1988), cellulase (Farkas *et al.*, 1985), urease and gelatin degradation and hydrogen sulphide production were also studied following the protocol as reported by Cappuccino and Sherman (1992).

Molecular characterization

Extraction of actinomycetes DNA and PCR amplification. Genomic DNA was extracted from eight selected *Streptomyces* isolates (having plant growth promoting properties) following the modified protocol of Boudjella *et al.* (2006). The purity check of DNA for analysing its quality and quantity was done by measuring the absorbance at 260 and 280 nm by spectrophotometer. The 16S rRNA gene fragment for the *Streptomyces* was amplified by using two universal primer pair fD1 (5'-GAGTTTGATCCTGGCTCA-3') and Rp2 (5'-CGGCTACCTTGTACGACTT-3'). The 16S rRNA was amplified by PCR using Promega kit.

The final volume of reaction mixture of 50 µl contained 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9.0 at 25°C), 1.5 mM MgCl₂, 200 mM of each dNTP, 1 mM of each primer, 0.25 U of *Taq* polymerase and 500 ng of template DNA. The amplification was performed on BioRad thermal cycler (initial denaturation step at 98°C for 3 min, after which *Taq* polymerase was added, followed by 30 amplification cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 10 min).

Amplified ribosomal DNA restriction analysis (ARDRA). For restriction analysis, amplified ribosomal DNA was digested with three restriction endonucleases *Taq* I, *Msp* I and *Hae* III (Promega, India) according to manufacturer's instructions and analyzed by horizontal electrophoresis in 2.5% agarose gels at 70 V for 2.5 h and documented on alpha-Imager gel documentation system (Alpha-Imager, USA). The restriction analysis profiles generated, were compared by calculating Jaccard's similarity coefficient for each pairwise comparison and dendrogram was constructed from the similarity matrix by the UPGMA. In order to test the goodness of fit of cluster analysis, co-phenetic value matrices were calculated and compared with the original similarity matrices that were UPGMA clustered by using the NTSYSpc analysis package (version 2.02e; Exeter Software, Setauket, NY). The amplified product of representative isolates from each clusters were purified by PCR purification kit (Promega, India) and sequenced directly with the *Taq*-mediated dideoxy chain terminator cycle sequencing in ABI 3130xl automated genetic analyser (Applied Biosystem, UK) according to manufacturer's instructions. The sequences were aligned by ClustalW and BlastN programme was used to compare the sequences deposited in public databases and the phylogenetic tree was constructed with the MEGA software version 4.1 (Saitou and Nei, 1987). Gaps were treated by pairwise deletions and bootstrap analysis was done by using 5,000 pseudo-replications.

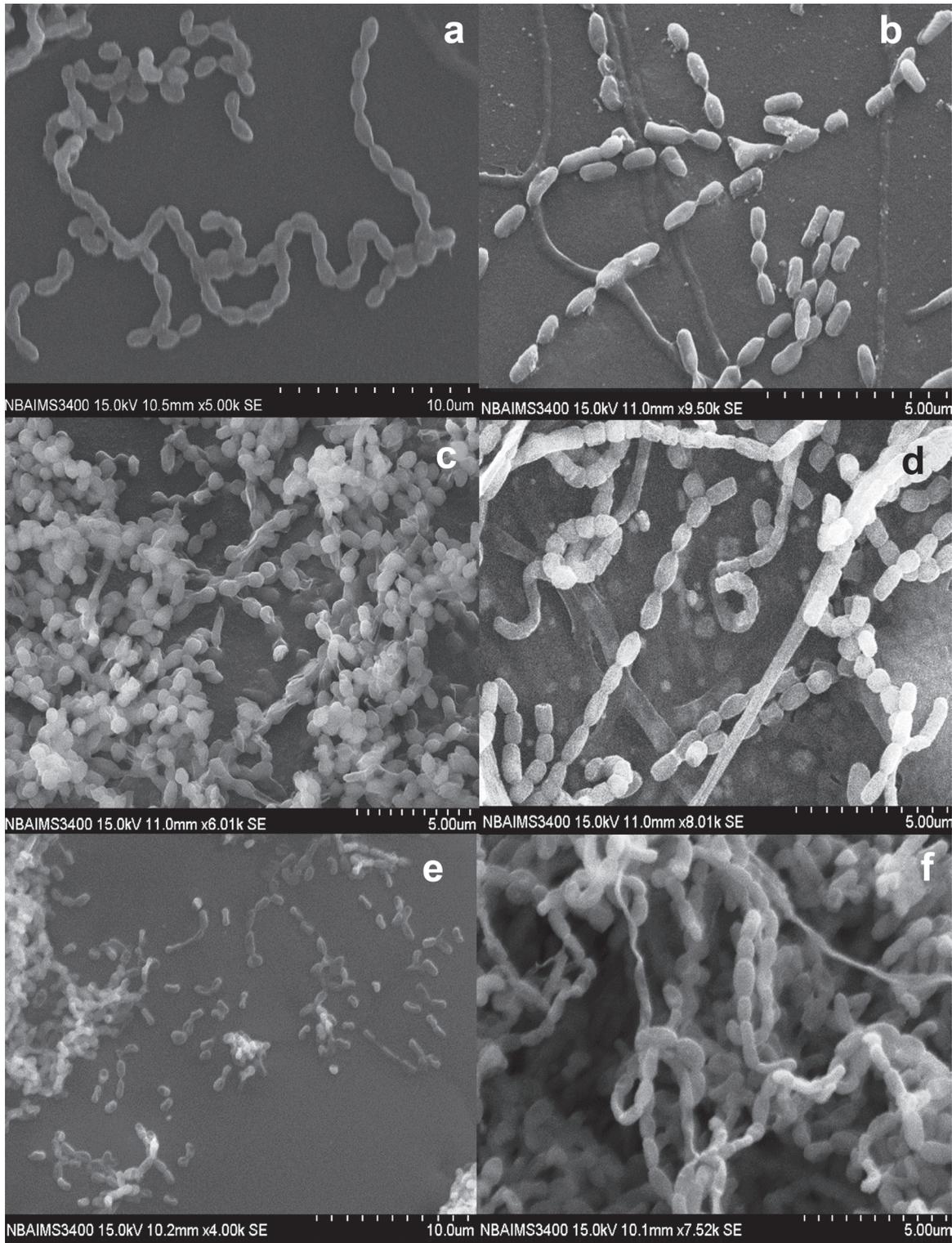
Accession numbers: A total of eight sequences of 16S rRNA gene were deposited in public databases (GenBank, NCBI) under the accession numbers from KC511801 to KC511808.

Results

Isolation and clustering of actinomycetes by polyphasic taxonomy. A total of 66 actinomycetes isolates were isolated from Coringa mangrove ecosystem, of these eight isolates showed considerable amount of variations in their colony morphology and possessing plant growth promoting attributes were chosen for further physiological and biochemical characterization. The population count of actinomycetes fluctuated from 19×10^4 to 80×10^4 g soil⁻¹ (Table I). Most of the isolates

belonged to genus *Streptomyces* and were tentatively identified by morphological characterization using aerial mycelial colour, substrate mycelial colour, pigments, arrangement of spores in chain, like straight chain, rectiflexibles *etc.* as revealed by scanning electron microscopy (Fig. 1). Cell wall composition analysis of actinomycetes using thin layer chromatography (TLC) revealed type I cell wall with LL-DAP isomers.

Physiological characterization of the isolated actinomycetes. Physiological tests are an important tool for classification of actinomycetes, influencing their growth and other properties. Physiological parameters like pH, temperature, NaCl concentration, carbon source utilization in the growth media were analysed. Growth of the selected 8 *Streptomyces* isolates occurred in the pH range of 6–9 with optimum growth at pH 7.



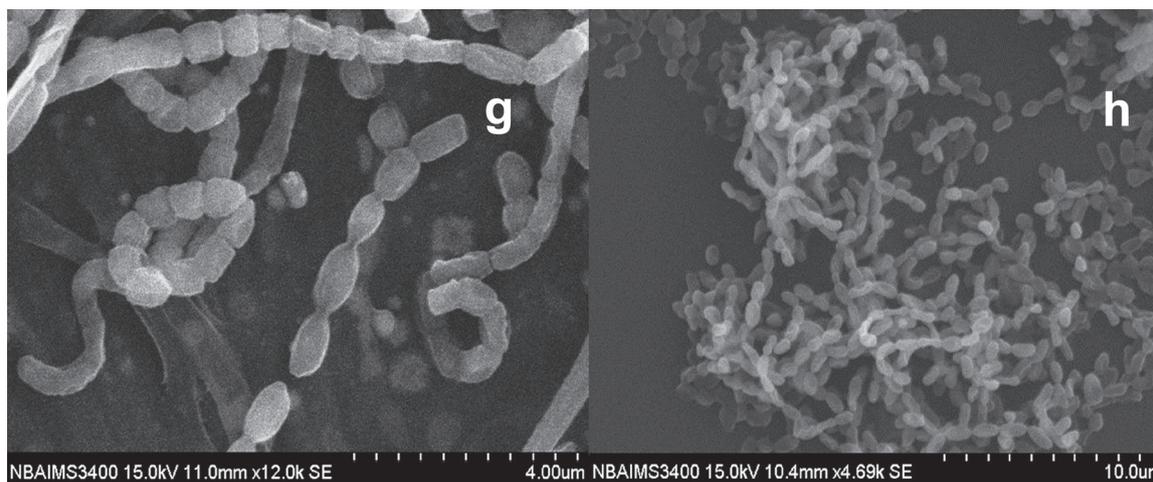


Fig. 1. Scanning electron microscopy (SEM) of *Streptomyces* isolates isolated from mangrove of Andhra Pradesh, India, showing variations in spore chain morphology.

a) AM2-2 (*Streptomyces globisporus*), (b) AM2-3 (*S. roseoviolaceus*), (c) AM2-4 (*S. cavourensis*), (d) AM2-10 (*S. celluloflavus*), (e) AM2-11 (*S. albogriseolus*) (f) AM2-7 (*S. spiralis*), (g) AM2-10 (*S. macrosporeus*) (h) AM5-16 (*S. rochei*)

The temperature range for growth was 20–45°C with the optimum temperature being 35°C. The isolates exhibited salt tolerance up to 8% with optimum growth at 6% NaCl; hence, the isolates could be placed in intermediate salt tolerance group. All the isolates were able to utilize 8–10 sugars as a carbon source out of 10 sugars being tested (Glucose, arabinose, rhamnose, mannitol, dulcitol, raffinose, fructose, sucrose, lactose and inositol).

Plant growth promoting attributes and extracellular enzyme production. Plant growth promoting and extracellular enzyme activity of the selected eight isolates revealed that all the 8 isolates (100%) were siderophore and IAA producers, 4 (50%) were phosphate solubilizers, 3 (37.5%) were H₂S and amylase producers, 5 (62.5%) were urease, protease and HCN producers, 6 (75%) of the selected isolates were nitrate reducing and only 1 (12.5%) isolate produced the enzyme gelatinase (Table II).

Molecular characterization and phylogenetic analysis. The molecular characterization of the selected *Streptomyces* isolated from mangroves were carried, based upon 16S rRNA gene amplification and its RFLP pattern with a set of three restriction enzymes *TaqI*, *MspI* and *HaeIII* (Fig. 2) and dendrogram (Fig. 3) was generated using NTSYSpc software. For More precise identification the isolates were sequenced by Sanger's di-deoxy nucleotide sequencing method and identified based on percentage similarity (> 97% compared with public database sequences, NCBI), by BLAST homology (Table III). Further phylogenetic analysis of the isolates was carried out for their similarity to known actinobacteria aligned together with the sequences (closest representatives), available in public databases (GenBank, NCBI), of actinobacteria (Fig. 4). Three genetic groups were formed among the identified

isolates (Table III). The 4 isolates included in group I showed 100% sequence identity compared with most closely related sequences in public database (*Streptomyces cavourensis*, *Streptomyces albogriseolus*, *Streptomyces spiralis*, *Streptomyces rochei*), followed by 2 isolates in group II with 99% similarity (*Streptomyces roseoviolaceus*, *Streptomyces celluloflavus*) and 2 isolates in group III showing < 99% similarity (*Streptomyces globisporus*, *Streptomyces macrosporeus*).

Discussion

Actinomycetes population have been identified as one of the prominent group of soil microbes which differ with soil type, soil pH, geographical location and climatic condition (Arifuzzaman *et al.*, 2010). The characterization of these microbes is as important as studying their existence in the natural environments (Hirsch and Valdes, 2010). Actinomycetes play a vital role in the soil such as mineralization of organic matters, immobilization of nutrients, antibiosis and production of plant growth promoters (Adegboye and Babalola, 2012). In our studies heat pre-treatments, enrichment techniques and selective isolation media resulted in considerable decrease in the unwanted bacterial population, which was similar as obtained by Hayakawa (2008). By various enrichment techniques and media used a total of 66 actinomycetes were isolated out of which 8 isolates with significant plant growth promotory attributes and growth under different pH and salinity levels, were characterised by 16S rRNA sequencing as belonging to *Streptomyces* genera. This genus had also been previously evaluated for the characterization of plant growth promoting and other important traits as

Table II
Phenotypic characteristics of all selected *Streptomyces* strains.

Properties	<i>S. globisporus</i> AM2-2	<i>S. roseoviolaceus</i> AM2-3	<i>S. cavourensis</i> AM2-4	<i>S. celluloflavus</i> AM2-10	<i>S. albogriseolus</i> AM2-11	<i>S. spiralis</i> AM2-7	<i>S. macrosporeus</i> AM2-8	<i>S. rochei</i> AM5-16
Morphological Characteristics								
Color of aerial mycelium	White	white	Ivory	White	White	White	White	Creamish white
Color of substrate mycelium	Yellow	Dark brown	Yellow brown	Light yellow	Yellow brown	Cream	Red brown	Yellow
Spore mass	Light brown	White	Ivory	Off white	Light grey	Cream	Creamish	Cream
Diffusible pigments	-	-	-	-	-	-	-	-
Carbon Source Utilization								
Glucose	+	+	+	-	+	+	+	+
Arabinose	-	+	+	+	-	+	+	+
Rhamnose	+	+	+	+	+	+	+	-
Mannitol	+	+	+	-	+	+	+	-
Dulcitol	-	+	+	+	-	+	+	+
Raffinose	+	+	+	+	+	+	+	+
Fructose	+	+	-	-	-	+	-	-
Sucrose	+	+	+	-	-	-	+	+
Lactose	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+
Chemotaxonomic characters								
Cell wall amino acid Analysis	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP	L-DAP
PGP traits								
IAA Production	+	+	+	+	+	+	+	+
Phosphate Solubilization	-	+	+	-	-	+	-	+
Siderophore Production	+	+	+	+	+	+	+	+
HCN Production	-	+	-	-	+	+	+	+
Nitrate Reduction	+	+	-	+	+	-	+	+
Hydrolytic enzymes and biochemical characterization								
Urease	+	-	-	+	+	-	+	+
Protease	-	+	-	+	+	-	+	+
Gelatinase	-	-	+	-	-	-	-	-
Amylase	+	-	-	-	-	+	-	+
H ₂ S Production	-	-	+	+	-	-	+	-

Table III
Closest BLASTN matches for the full 16S rRNA sequences and their percentage similarity with the closest actinobacterial strains.

IsolateCode	Closest species	GenBank accessionnumber	Similarity (%)
AM2-2	<i>Streptomyces globisporus</i>	KC511801	98%
AM2-3	<i>Streptomyces roseoviolaceus</i>	KC511802	99%
AM2-4	<i>Streptomyces cavourensis</i>	KC511803	100%
AM2-10	<i>Streptomyces celluloflavus</i>	KC511804	99%
AM2-11	<i>Streptomyces albogriseolus</i>	KC511805	100%
AM2-7	<i>Streptomyces spiralis</i>	KC511806	100%
AM2-8	<i>Streptomyces macrosporeus</i>	KC511807	98%
AM5-16	<i>Streptomyces rochei</i>	KC511808	100%

Fig. 2. Restriction digestion banding pattern of isolates amplified by 16S rDNA and digested by *Taq* I, *Msp* I and *Hae* III restriction enzymes.

Lane M: 100bp molecular weight marker (Genei, Bangalore), Lane 1: AM2-2, Lane 2: AM2-3, Lane 3: AM2-4, Lane 4: AM2-10, Lane 5: AM2-11, Lane 6: AM2-7, Lane 7: AM2-8 and Lane 8: AM5-16

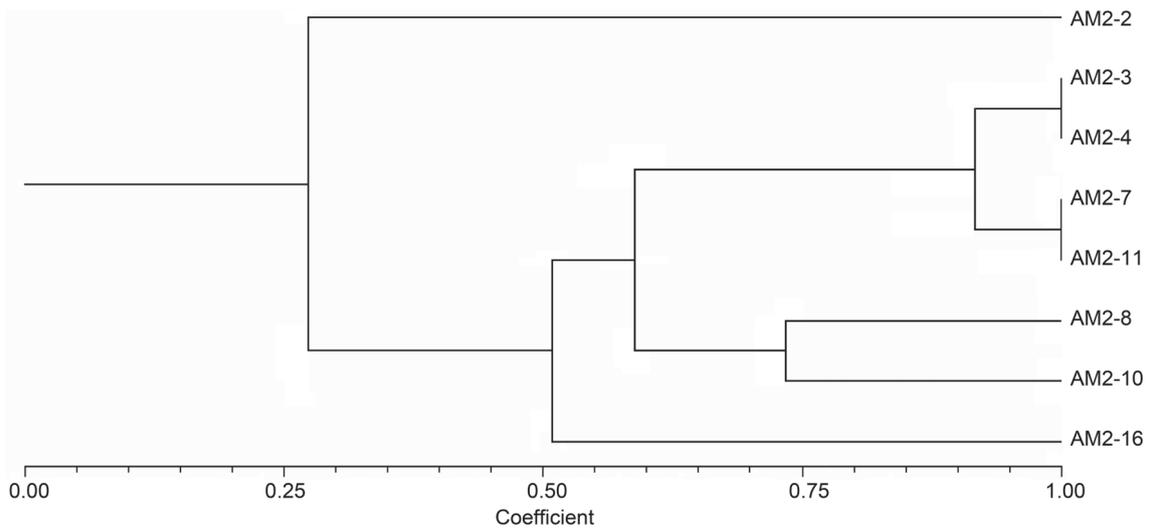
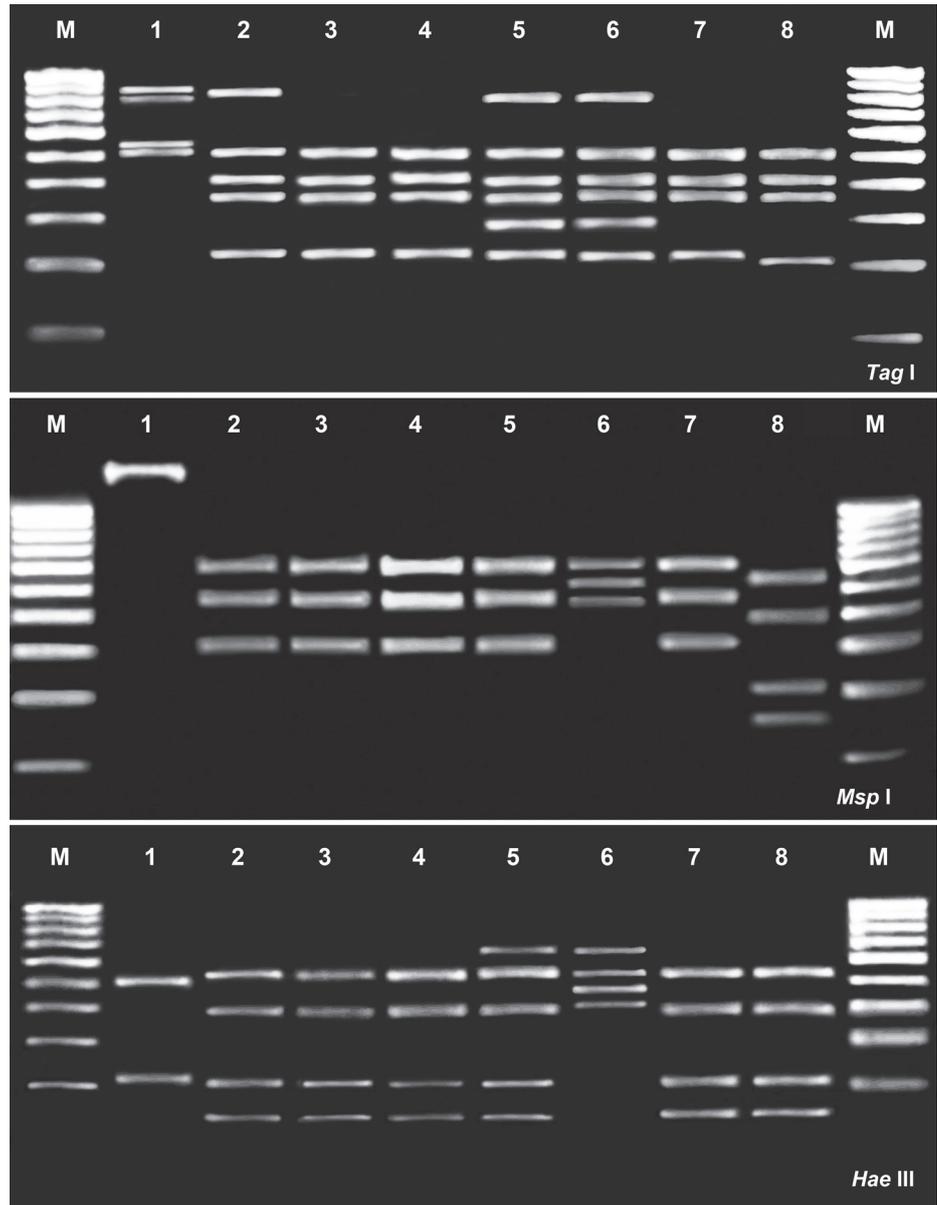


Fig. 3. UPGMA cluster analysis showing the genetic relationship among eight isolates of Actinomycetes, banding pattern based on restriction digestion by restriction enzyme *Taq* I, *Hae* III and *Msp* I

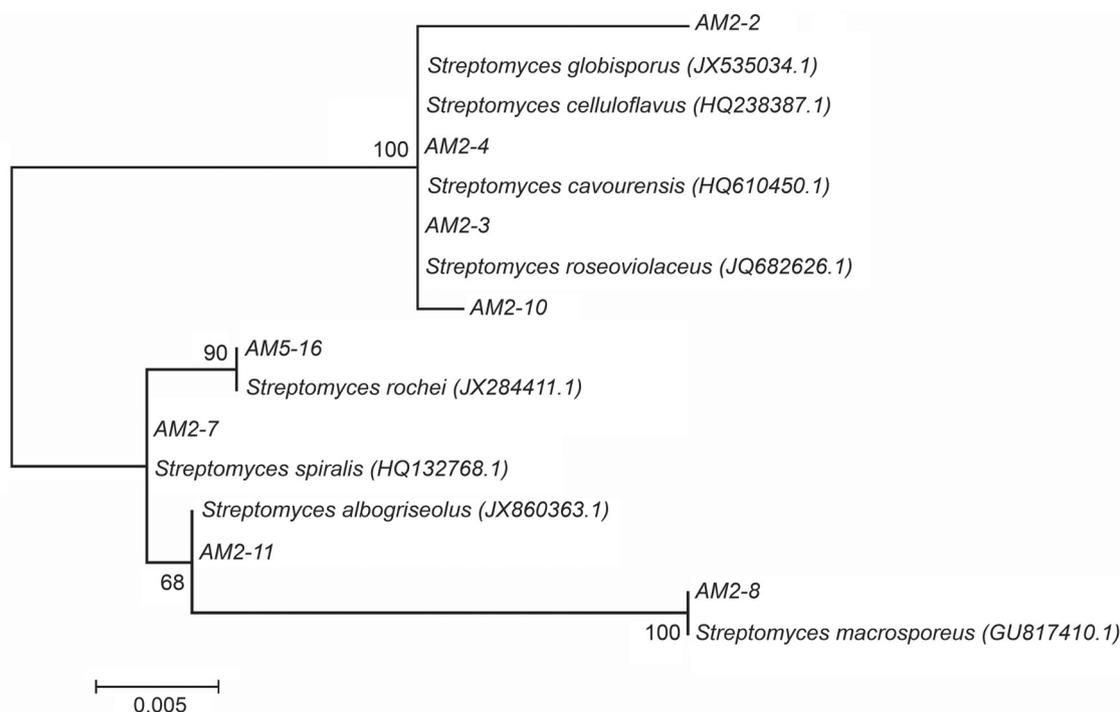


Fig. 4. NJ phylogenetic tree of full 16S rRNA sequences from selected isolates. The sequence data for several closely related actinobacterial type cultures were recovered from GenBank and included in the tree. The boot strap values from 5,000 pseudoreplications are shown at each of the branch points on the tree. Bar indicates % similarity

enough information available in the public databases (Zhang *et al.*, 1998; Malviya *et al.*, 2011; Yandigeri *et al.*, 2012), but *Streptomyces* isolated from stressed habitats with plant growth promoting potential have not been studied extensively. Bacteria isolated from different stressed habitats possess stress tolerance capacity alongwith plant growth-promoting traits. Salinity tolerance and the ability of *Streptomyces* for the production of extracellular enzymes, plant growth promoting attributes and other activities were evaluated and it was reported that all the 8 (100%) *Streptomyces* isolates from mangrove region could tolerate up to 8% NaCl concentration showing optimum growth at 6% NaCl concentration, hence the strain could be placed in intermediate salt tolerance group according to Tresner *et al.* (1968). The ability of *Streptomyces* strains to tolerate high concentrations of NaCl is well known (Waksman, 1959). Similar results were reported from the *Streptomyces* isolated from Indo-Gangetic Plains (IGP) (Malviya *et al.*, 2011), and halophilic *Streptomyces* from India and their utilization in agricultural fields (Vasavada *et al.*, 2006). Da Silva Sousa *et al.* (2008) studied six *Streptomyces* isolated from rhizospheric soil of various crops and found out that they could tolerate a NaCl level up to 3%. Kavya *et al.* (2012), also reported salinity tolerance of *Streptomyces* from Coringa mangrove ecosystem but their results indicated a tolerance of 3–4% NaCl concentration, which is less as compared to isolates under current study. Sadeghi *et al.* (2012)

have reported the beneficial role of *Streptomyces* on PGP activity under salinity stress. Hence, it can be concluded that these strains may have the ability to survive in the harsh environments such as saline and acidic to alkaline pH soils. In our studies all the 8 *Streptomyces* isolates were positive IAA and siderophore producers. There are many reports which demonstrated the ability of endophytic and rhizospheric soil *Streptomyces* to produce indole acetic acid and thus promote plant growth (Khamna *et al.*, 2010; Solans *et al.*, 2011; Yandigeri *et al.*, 2012; Kaur *et al.*, 2013). In the rhizosphere soils, root exudates are the natural source of tryptophan for rhizosphere micro-organisms, which may enhance auxin (IAA) biosynthesis in the rhizosphere. Likewise Sadeghi *et al.* (2012) have also reported the production of IAA and siderophore by halotolerant *Streptomyces* isolate. Siderophore production may be involved in the inhibition of pathogens and thus promote plant growth indirectly because *Streptomyces* species are known for the production of hydroxamate type siderophores, which inhibit phytopathogen growth by competing for iron in rhizosphere soils (Khamna *et al.*, 2009; Kaur *et al.*, 2013), thus our isolates could be involved in the inhibition of pathogens. Malviya *et al.* (2011), also reported production of siderophore by *Streptomyces* isolated from IGP region but in their studies only 8% of the isolates from the total 145 isolates were positive siderophore producers but in our studies 75% of the selected *Streptomyces* were nitrate

reducers, 50% were phosphate solubilizers and 62.5% were HCN producers. In agriculture, biological phosphate solubilisation as an alternative to natural phosphate utilisation plays an important role in efficient nutrient uptake of plants. Patil *et al.* (2010) isolated nine antagonistic actinomycetes from IGP region and found that out of them 8 were *Streptomyces*. Out of these eight *Streptomyces* isolates five were phosphate solubilizers and one produced HCN. Some actinomycetes are known to produce hydrolytic enzymes such as protease and amylase (Kaur *et al.*, 2013). These enzymes help in preventing the crops from plant pathogens and deleterious microbes by degrading their cell walls. In our study we reported that five of our isolates could produce enzyme protease and three isolates were amylase producers. Also it was reported that five isolates were producing urease, three H₂S producers and one isolate was able to show gelatinase activity. After studying the cultural and morphological characteristics of the potent plant growth promoting isolates, they were assigned under the genus *Streptomyces*. Concerning phenotypic characteristics, isolates produced varied colour aerial and substrate mycelia. Their chemotaxonomic characteristics further validate that they all belong to genus *Streptomyces* as their cell wall contain L-diaminopimelic acid (cell wall type-I). Thus, this study is in accordance with the previous reports that *Streptomyces* are known to be predominant among actinomycetes with antagonistic and plant growth promoting potential and produce antifungal compounds (Ouhdouch *et al.*, 2001; Kaur *et al.*, 2013).

The reliable generic identification of members of this genus by the first approach was confirmed by DNA sequence analysis. Variations in 16S rDNA can also be assessed by analyzing restriction fragment length polymorphism (RFLP) of 16S rDNA sequences. PCR based markers such as RAPD, RFLP, DAPD and SSR have been used to discriminate bacterial strains and to analyse genetic diversity (Yadav *et al.*, 2013). In this study, we used 16S rDNA sequence-based phylogenetic analysis to investigate the species diversity of *Streptomyces*. It is also known that microbes with 16S rRNA sequence similarity up to 97% identical should be considered as members of the same genus (Petit *et al.*, 1999; Malviya *et al.*, 2011). Since all the isolates were belonging to genus *Streptomyces*, we tried to study their diversity using ARDRA, which is the rapid and convenient method, and can be very useful in grouping actinobacterial isolates efficiently as well as effectively reduce the number of isolates by de-replicating the isolates during screening for diversity (Jiang *et al.*, 2010). In the current study, we used a set of three restriction endonucleases *Msp* I, *Hae* III and *Taq* I that specifically recognize the sequence 'CCGG', 'GGCC' and 'TCGA' respectively. The results of the different RFLP patterns obtained allowed

us to effectively differentiate the strains into distinct groups of actinobacteria. In our study, a total of 8 representative isolates were identified by 16S rRNA gene sequence analysis as *S. globisporus* (KC511801), *S. roseoviolaceus* (KC511802), *S. cavourensis* (KC511803), *S. celluloflavus* (KC511804), *S. albogriseolus* (KC511805), *S. spiralis* (KC511806), *S. macrosporeus* (KC511807) and *S. rochei* (KC511808). All the isolates recorded 98–100% similarity with the representative isolates as shown in the Table III. Similar results were observed by Patil *et al.* (2010) and Malviya *et al.* (2011) the *Streptomyces* isolated by them also showed 98–100% similarity except one isolate in case of Malviya *et al.* 2011 which was 96% similar with the representative isolate.

The present study highlights that mangroves are the potential reservoirs for actinomycetes especially which can tolerate salt levels and biochemical and plant growth promotion assay of the isolates demonstrates their potential to be used as biocontrol agents and biofertilizers which may play important role in plant growth promotion either directly or indirectly and thereby increase crop yield.

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Germination and Inactivation of *Alicyclobacillus acidoterrestris* Spores Induced by Moderate Hydrostatic Pressure

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Abstract

Given the importance of spoilage caused by *Alicyclobacillus acidoterrestris* for the fruit juice industry, the objective of this work was to study the germination and inactivation of *A. acidoterrestris* spores induced by moderate hydrostatic pressure. Hydrostatic pressure treatment can induce the germination and inactivation of *A. acidoterrestris* spores. At low pH, spore germination of up to 3.59–3.75 log and inactivation of 1.85–2.04 log was observed in a low pressure window (200–300 MPa) applied at 50°C for 20 min. Neutral pH suppressed inactivation, the number of spores inactivated at pH 7.0 was only 0.24–1.06 log. The pressurization temperature significantly affected spore germination and inactivation. The degree of germination in apple juice after pressurization for 30 min with 200 MPa at 20°C was 2.04 log, with only 0.61 log of spores being inactivated, while at 70°C spore germination was 5.94 log and inactivation 4.72 log. This temperature strongly stimulated germination and inactivation under higher (500 MPa) than lower (200 MPa) pressure. When the oscillatory mode was used, the degree of germination and inactivation was slightly higher than at continuous mode. The degree of germination and inactivation was inversely proportional to the soluble solids content and was lowest in concentrated apple juice.

Key words: *Alicyclobacillus acidoterrestris*, germination, high hydrostatic pressure, inactivation spores

Introduction

Alicyclobacillus acidoterrestris, thermoacidophilic and spore-forming bacteria may cause spoilage of pasteurized juices and beverages, producing compounds associated with a disinfectant-like odour: guaiacol, 2,6-dibromophenol, 2,6-dichlorophenol (Baumgart *et al.*, 1997; Borlinghaus and Engel, 1997; Pettipher *et al.*, 1997; Orr *et al.*, 2000; Jensen and Whitfield, 2003; Gocmen *et al.*, 2005; Niwa, 2005; Danyluk *et al.*, 2011).

These bacteria have been isolated from orchard soil (Eguchi *et al.*, 2001; Goto *et al.*, 2008; Groenewald *et al.*, 2008; Wang *et al.*, 2010), fruits (Eguchi *et al.*, 2001; Parish and Goodrich, 2005), juice production environment (Eguchi *et al.*, 2001; Steyn *et al.*, 2011, Zhang *et al.*, 2013) and from many final products—juices and juice concentrates, all over the world (Cerny *et al.*, 1984; Splittstoesser *et al.*, 1994; Baumgart *et al.*, 1997; Pettipher *et al.*, 1997; Eguchi *et al.*, 2001; Durak *et al.*, 2010; McKnight *et al.*, 2010; Danyluk *et al.*, 2011; Oteiza *et al.*, 2011).

The presence of this new type of spoilage bacterium in aseptically packaged apple juice was first reported

in 1984 (Cerny *et al.*, 1984) and since then *A. acidoterrestris* has been recognized as a significant spoilage organism in the fruit juice industry (Silva *et al.*, 2000).

A. acidoterrestris strains show the ability to germinate and grow at a pH range of from 2.0 to 6.0 at a temperature of 20–55°C, with an optimum range of 42–53°C (Baumgart *et al.*, 1997; Deinhard *et al.*, 1987; Sokołowska *et al.*, 2010). *A. acidoterrestris* contains ω -cyclohexyl fatty acids in its cellular membrane composition. These ring structures are of special physiological importance for cells at a high growth temperature and low pH (Kirschke and Poralla, 1990).

A. acidoterrestris spores show extremely high thermal resistance depending on the kind of juice, its soluble solids content and pH. The values of D_{95} (time in minutes, during which the number of living cells decrease by 90%, at 95°C) in various juices that can be found in the literature were 1.85–15.1 min (Splittstoesser *et al.*, 1994; Baumgart *et al.*, 1997; Komitopolou *et al.*, 1999; Silva *et al.*, 1999; Bahceci and Acar, 2007; Sokołowska *et al.*, 2008; Bevilacqua and Corbo, 2011). The standard pasteurization process using temperatures

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of 85–95°C, which is aimed at destroying vegetative pathogens, is therefore ineffective against these bacteria spores (Splitstoesser *et al.*, 1994; Baumgart *et al.*, 1997; Silva *et al.*, 2000).

Using a higher temperature can negatively affect the nutritious and sensory quality of the juices, therefore there are attempts to use hydrostatic pressure (HP) as a non-thermal spore-inactivating process.

A few studies report *A. acidoterrestris* vegetative cell and spore inactivation by HP (Lee *et al.*, 2002; Alpas *et al.*, 2003; Ardia, 2004; Lee *et al.*, 2006; Vercammen *et al.*, 2012; Silva *et al.*, 2012; Skąpska *et al.*, 2012, Sokołowska *et al.*, 2012, Sokołowska *et al.*, 2013). *A. acidoterrestris* vegetative cells were killed by HP when 350 MPa at 50°C was used. More than a 4 log reduction was achieved in BAM broth (*Bacillus acidocaldarius* medium), orange, apple and tomato juices after 20 min pressurization (Alpas *et al.*, 2003).

It was also shown that the effect of *A. acidoterrestris* spore inactivation in apple juice, using pressure ranging from 207 to 621 MPa (up to 10 min), was strongly dependent on the process temperature: at 22°C no reduction was achieved, at 45°C a max. 3.5 log reduction occurred and at 70°C and at 90°C, complete (>5.5 log) reduction was observed after 5 min treatment, irrespective of the pressure used (Lee *et al.*, 2002). In accordance with these results are those of Ardia (2004).

Previously a study by Skąpska *et al.* (2012), showed large differences in sensitivity to HP between the spores of eight wild *A. acidoterrestris* strains. The reduction in the spore number in apple juice after treating at 300 MPa for 10 min was 1.3–3.5 log, depending on the strain. Increasing the pressure to 500 MPa did not result in a significantly more efficient pasteurization process. The use of oscillatory high pressure has been proven to be more effective. The greatest reduction in spores of the two most resistant to HP *A. acidoterrestris* strains (TO-29/4/02 and TO-117/02) was 2.4 and 3.1 log cfu/ml when 300 MPa in six five-min cycles at 50°C were applied. Subsequent research (Sokołowska *et al.*, 2012) has shown that lower pressure of 200 MPa at 50°C, applied both in a continuous and oscillatory mode, produced an even better effect. In these conditions, a reduction of 1.5 log in the *A. acidoterrestris* (TO-29/4/02 strains) spore count in apple juice was obtained after 10 min of continuous pressurization. After six five-min cycles a reduction of 5.0 log was achieved.

A recent study carried out using orange juice (Silva *et al.*, 2012), showed an approximate 2 log reduction in *A. acidoterrestris* spores after processing with 200 MPa at 65°C for 10 min, slightly better results (~2.5 log) were achieved when the pressure was increased to 600 MPa.

Only one article concerning the germination of *A. acidoterrestris* spores induced by HP was found (Vercammen *et al.*, 2012). This experiment, carried out

for 10 min with a pressure of 100–600 MPa, in buffers at pH 4.0, 5.0 and 7.0 and a temperature of 40°C, showed no significant spore inactivation, although spore germination of up to about 2 log was observed in a low pressure window (100–300 MPa). When spores were treated in tomato sauce with pH 4.2 and 5.0 with 100–600 MPa at 25, 40 and 60°C for 10 min, the germination level was generally higher than in buffers. HP treatment conducted at 60°C resulted in the inactivation of most of the germinated spores.

The inactivation of *A. acidoterrestris* spores under high pressure was shown to be suppressed by a high soluble solids content in apple juice concentrates (Lee *et al.*, 2006; Sokołowska *et al.*, 2013). No information about the germination of these spores in concentrated juices was found.

Depending on the temperature and level of pressure applied, bacterial endospores pass through different physiological pathways, which could induce spore germination or their subsequent inactivation during treatment. Moderate hydrostatic pressure induces spore germination by triggering the spores' nutrient receptors (Setlow, 2003; Reineke *et al.*, 2012). During the germination process, spores progressively lose their typical resistance and become more readily inactivated like vegetative cells (Wuytac *et al.*, 1998; Setlow, 2003; Moir, 2006; Luu and Setlow, 2014). The changes in spore sensitivity to heat and high pressure, which were used to differentiate the stages in the germination process in this work, were described by Black *et al.* (2007). In the first stage, the spores partially lose their impermeability to water, leading to an influx of water (with a slight increase in volume) and leakage of solutes (dipicolinic acid – DPA, Ca²⁺). Consequently, they become sensitive to wet heat (Setlow, 2003). During the second stage, the cortex is enzymatically digested, leading to full core rehydration, greater hydration of the core macromolecules, and a greater loss of spore-specific resistance, including to high pressure (Wuytac *et al.*, 1998). At the end of the second stage, the small acid-soluble spore proteins (sasP) are hydrolyzed to amino acids, which are subsequently used in protein synthesis by the growing cell (Moir, 2006; Setlow, 2003). Protein synthesis and spore metabolism only occur in the outgrowth phase, in which the germinated spore is converted into a growing cell. According to Wuytac *et al.*, 1998 and Reineke *et al.* (2012) spores are unable to proceed to stage two of germination above 500 MPa. Further spores are unable to outgrowth under pressure.

A treatment pressure above 500 MPa combined with elevated temperatures (>60°C), could induce rapid spore germination by opening the spores' Ca²⁺-DPA channels (Paidhungat *et al.*, 2002), which is accompanied by the release of large depots of DPA and the associated divalent cations (predominantly Ca²⁺) from

their core. The presumed direct opening of Ca²⁺-DPA channels are even active at 200 MPa and a moderate temperature, but this is not a dominant factor influencing the germination rate (Reineke *et al.*, 2012).

The aim of this work was to study the germination and inactivation of *A. acidoterrestris* spores induced by moderate HP and the effect of different factors, such as pressure, temperature, time, mode of pressure application, type of medium and soluble solids content in apple juice, on this process. Knowledge of factors that promote the germination step may lead to the increased lethality of HP treatments on bacterial spores.

Experimental

Materials and Methods

Tested organism. The *A. acidoterrestris* strain TO-117/02 used in this study was isolated from Polish concentrated apple juice, using the International Federation of Fruit Juice Producers' method. Confirmation of *A. acidoterrestris* was based on the utilization of erythritol, with acid production (Baumgart, 2003) and guaiacol production in YSG medium with vanillic acid (Niwa and Kawamoto, 2003). Identification at the species level was also performed by 16S *rRNA* gene sequencing and 16S *rRNA* gene RFLP characterization (Dekowska *et al.*, 2013). This strain was chosen from among eight wild strains as highly resistant to HP in our previous study (Skąpska *et al.*, 2012). Spores were produced based on the method described by Massaguer *et al.* (2002), with some modifications (Sokołowska *et al.*, 2012).

Just before the experiments, spores were suspended either in McIlvain buffer solution (mixture of relevant volume of 0.1 molar citric acid and 0.2 molar disodium phosphate) pH 4.0 and pH 7.0 or in apple juice at approximately 6–7 log cfu/ml.

Hydrostatic pressure treatment. Treatment was carried out in a high pressure food processor piston type vessel with inner diameter 110 mm, a working volume of 1.5 l, with a maximum operating pressure of 600 MPa (Izopress, Moscow). The pressure-transmitting fluid was distilled water and polypropylene glycol (1:1). The working temperature of the apparatus was 0–50°C. Pressure of up to 600 MPa was generated in 15–20 s; the release time was 15 s.

Thirteen millilitre samples in polyethylene tubes (Sarstedt®) were exposed to high pressure treatment in a continuous or oscillatory mode with 100, 200, 300, 400 or 500 MPa at a temperature of 20 or 50°C. Each cycle consisted of 5 min holding time at an elevated pressure and a 5 min pause at atmospheric pressure. The temperature was measured in the transmitting fluid and the increase during compression was 2°C/100 MPa.

For experiments at 70°C, U 4000/65 (Unipress) apparatus was used. The volume of the treatment chamber was 0.95 l and the maximum pressure 600 MPa. The pressure-transmitting fluid used was also distilled water and polypropylene glycol (1:1). The working temperature of the apparatus ranged from –10°C to +80°C. A pressure of up to 200 MPa was generated in 120–150 s; the release time was 2–4 s.

Unpressurized samples were used as controls. The pressurization times reported do not include the come-up and come-down time. The assays were performed using two independent samples from two independent processes.

Apple juice. Apple juice concentrate (70.7°Bx, 53.0% sugar, pH 3.1, titratable acidity as malic acid 3.89%), containing no *A. acidoterrestris* spores, was obtained from the Polish producer. Two-, three- and six-fold dilutions were made from this concentrate with sterile deionized water. If commercial pasteurized apple juice was used (pH 3.4, soluble solids 11.2°Bx), before conducting the experiment it was filtered through a 0.45 µm Millipore® filter to remove possible *A. acidoterrestris* spores. The soluble solids and pH were measured using a refractometer (MS REF 090L My-Soft) and pH meter (CP-315 ELMETRON).

Determination of inactivation and germination of *A. acidoterrestris* spores. The number of spores surviving the different HP treatments was evaluated immediately after processing and after heat treatment at 80°C for 10 min. This heat treatment was found not to kill ungerminated spores (data not shown). The spread plate method on BAT-agar (Merck) with incubation for 5 days at 45°C was used. Limit of detection this method was 1 cfu/ml.

Pressure-induced inactivation was the difference between the plate count before and after HP treatment. Pressure-induced germination was the difference between the plate count before HP treatment and after HP followed by heat treatment at 80°C for 10 min (Black *et al.*, 2007; Nguyen Thi Minh *et al.*, 2010; Vercammen *et al.*, 2012), expressed as log (cfu/ml).

Data analysis. An analysis of the variance and Duncan's multiple-range test, using StatSof Statistica 7.1, was used to test the significance of the differences ($p < 0.05$) between the number of germinated and inactivated spores. The bars on the figures indicate the mean standard deviation for data points.

Results and Discussion

Influence of pressure and type of medium on the germination and inactivation of *A. acidoterrestris* spores. To study the effect of moderate pressure on the germination and inactivation of *A. acidoterrestris*

Table I

The germination and inactivation of *A. acidoterrestris* spores under various pressures at low and neutral pH in buffer solutions and commercial apple juice (HP treatment at 50°C for 20 min).

Pressure [MPa]	McIlvain buffer pH 4.0		McIlvain buffer pH 7.0		Commercial apple juice	
	Germination log [cfu/ml]	Inactivation log [cfu/ml]	Germination log [cfu/ml]	Inactivation log [cfu/ml]	Germination log [cfu/ml]	Inactivation log [cfu/ml]
100	3.14 ± 0.03 ^{ba}	1.85 ± 0.13 ^{baA}	2.95 ± 0.02 ^{ab}	0.24 ± 0.09 ^{ab}	3.06 ± 0.03 ^{aA}	1.83 ± 0.06 ^{aA}
200	3.75 ± 0.18 ^{aA}	2.02 ± 0.02 ^{baA}	2.84 ± 0.34 ^{ab}	0.31 ± 0.04 ^{abB}	3.59 ± 0.10 ^{ba}	1.95 ± 0.12 ^{aA}
300	3.74 ± 0.05 ^{aA}	2.04 ± 0.05 ^{aA}	2.73 ± 0.07 ^{ab}	0.38 ± 0.06 ^{bb}	3.30 ± 0.01 ^{cC}	1.85 ± 0.06 ^{aA}
400	3.39 ± 0.06 ^{ba}	1.84 ± 0.06 ^{ba}	2.69 ± 0.09 ^{ab}	1.06 ± 0.03 ^{cb}	2.88 ± 0.02 ^{dB}	1.26 ± 0.03 ^{bc}
500	3.32 ± 0.12 ^{ba}	1.37 ± 0.05 ^{ca}	1.86 ± 0.02 ^{bb}	0.66 ± 0.01 ^{dB}	1.73 ± 0.01 ^{eb}	0.85 ± 0.02 ^{cc}

Mean values in columns with different lowercase letters are significantly different at $p < 0.05$, separately for germination and inactivation.

Mean values in rows with different capital letters are significantly different at $p < 0.05$, separately for germination and inactivation.

spores, a temperature of 50°C was chosen to stimulate germination without causing a thermal pasteurization effect. Low (4.0) and neutral (7.0) pH buffers and real food – apple juice – were used in this part of the study.

Germination of spores was observed in all used media (Table I). The highest germination, from 3.14 to 3.75 log, was found in the pH 4.0 buffer within the entire range of applied pressure. Statistically significant ($p < 0.05$) maximum germination occurred at 200 and 300 MPa in the pH 4.0 buffer. Similar germination was observed in apple juice pressurized at 200 MPa – 3.59 log. Generally the lowest germination was obtained in the pH 7.0 buffer, but the results noted in the pressure range 100–400 MPa did not differ significantly ($p > 0.05$).

Significant ($p < 0.05$) inactivation, of 1.84–2.04 log, was observed in the pH 4.0 buffer as a result of pressure treatment in the 100–400 MPa range. The level of inactivation in apple juice was similar – a reduction from 1.83 to 1.95 log was achieved in the slightly narrower 100–300 MPa range. Less inactivation occurred at 400 or 500 MPa,

A. acidoterrestris spores germinated in the pH 7.0 buffer but were not inactivated during HP treatment. Inactivation only reached 0.24–1.06 log, with a significant maximum ($p < 0.05$) at 400 MPa (Table I).

The results indicate that a low pH supports both the germination and inactivation of *A. acidoterrestris* spores. In our study the highest germination was achieved when 200–300 MPa in pH 4.0 buffer or 200 MPa in apple juice at 50°C was used, which is consistent with the results obtained by Vercammen *et al.* (2012).

Considerable inactivation of *A. acidoterrestris* spores was also achieved in our study. In apple juice in the low pressure window (100–300 MPa), the reduction was significantly higher than at 400 or 500 MPa. Contrary to our results, in the Lee *et al.* (2002) study there was no significant difference among the effect of 207, 414 and 621 MPa on *A. acidoterrestris* spore viability at 45°C in apple juice, a 3.5 log reduction was always observed,

irrespective of the pressure used. Yet other results were obtained by Silva *et al.* (2012) in orange juice. When processed at 45°C, the inactivation of spores treated with 200 MPa was only about 0.5 log and about 1.0 log, when 600 MPa was used. The different results obtained in the work presented may indicate large variations in pressure resistance among the *A. acidoterrestris* strains, as well as the influence of the kind of juice, sporulation conditions and equipment used in the various studies.

Impact of temperature and time on the germination and inactivation of *A. acidoterrestris* spores in apple juice during pressure treatment. For this study, pressure of 200 MPa was chosen as the best for germination and inactivation of *A. acidoterrestris* spores in apple juice. As shown in Figure 1, the germination of *A. acidoterrestris* spores in apple juice depended on the temperature and time. At 20°C there was little germination and 2.04 log was achieved after 30 min, while inactivation in these conditions achieved only 0.61 log. After 5 min at 50°C, the degree of germination was 2.65 log, but the spores did not inactivated. Prolonging the time of treatment at 50°C to 30 min, significantly ($p < 0.05$) supported both germination and inactivation, which resulted in 4.06 log of germinated spores and 2.76 log of inactivated spores.

When HP treatment was conducted at 70°C, germination was significantly higher ($p < 0.05$) than at 50°C for all used pressurization times. Most of the germinated spores were also inactivated at 70°C. Treatment at this temperature was found not to kill ungerminated spores, but to cause a thermal pasteurization effect on vegetative cells. Approx. 1.7 log of *A. acidoterrestris* TO-117/02 strain vegetative cells was inactivated after 30 min at 70°C (data not shown).

The highest germination and inactivation were achieved when 200 MPa was applied at 70°C, which is consistent with the results obtained by other researchers (Silva *et al.*, 2012; Vercammen *et al.*, 2012). Spores of the *A. acidoterrestris* TO-117/02 strain used in our study showed higher resistance to HP at 70°C than the

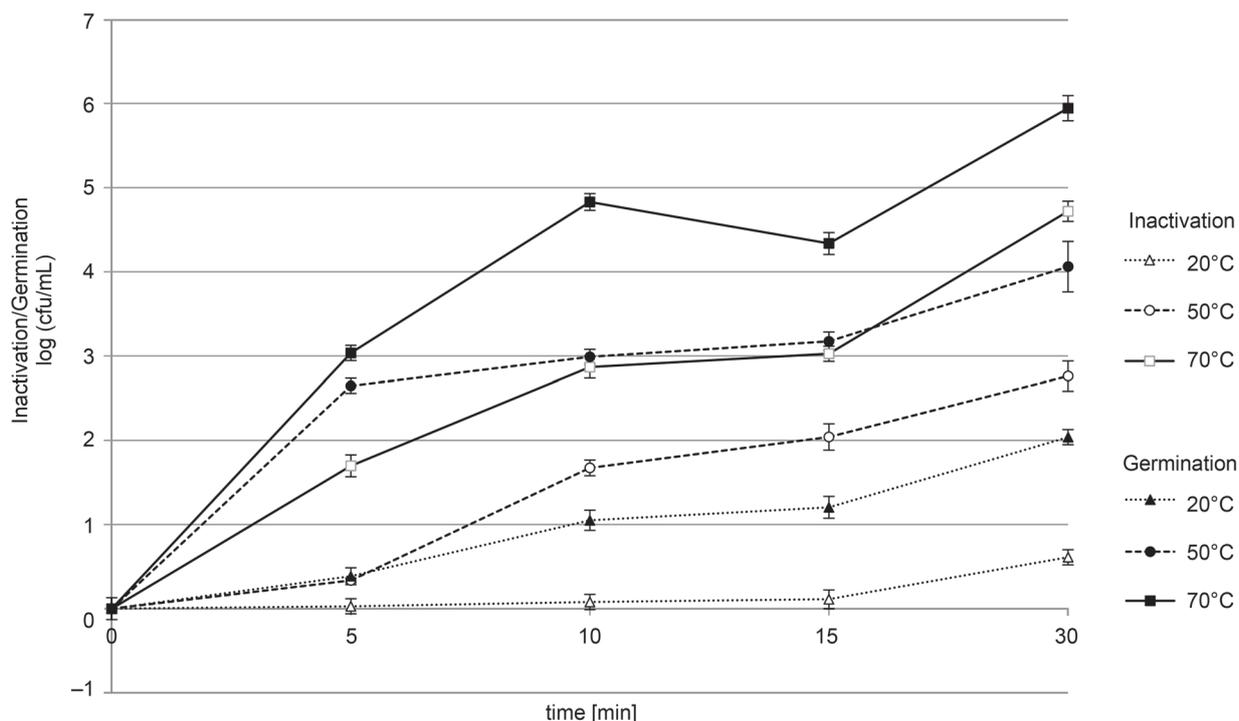


Fig. 1. Germination and inactivation of *A. acidoterrestris* spores treated with 200 MPa in commercial apple juice at various temperatures.

strain used by Lee *et al.* (2002), who observed a complete (>5.5 log) reduction in apple juice after 5 min treatment with 207 MPa at 71°C.

The higher spore inactivation rate at a higher temperature can be explained by the acceleration of enzymatic reactions during progression from the first to second stage of germination as well as by the fact that first stage germinated spores are directly inactivated by temperatures above 70°C (Nguyen Thi Minh *et al.*, 2010).

Since pressurization conducted at 500 MPa at 50°C resulted in the lowest germination and inactivation, it was also verified whether an increase in temperature would enhance these processes. A higher process temperature (70°C) strongly stimulated germination and inactivation at 500 MPa. The germination achieved

6.72 log and inactivation 6.13 log and the increase was significantly higher than at a lower (200 MPa) pressure (Table II). This phenomenon was also observed in tomato juice (Vercammen *et al.*, 2012).

Effect of mode of pressure application on the germination and inactivation of *A. acidoterrestris* spores. Samples of *A. acidoterrestris* spores in buffer solutions (pH 4.0 and 7.0) and apple juice were exposed to hydrostatic pressure treatment in continuous or oscillatory mode with 200 MPa at a temperature of 50°C. Each cycle was composed of 5 min holding time at an elevated pressure and a 5 min pause at atmospheric pressure.

The degree of germination and inactivation of *A. acidoterrestris* spores increased when the pressure time was prolonged and the pH decreased (Table III). After

Table II
Germination and inactivation of *A. acidoterrestris* spores after 20 min under various pressures and temperature in commercial apple juice.

Pressure [MPa]	Pressurization temperature			
	50°C		70°C	
	Germination log [cfu/ml]	Inactivation log [cfu/ml]	Germination log [cfu/ml]	Inactivation log [cfu/ml]
200	3.59 ± 0.10 ^{aA}	1.95 ± 0.12 ^{aA}	5.84 ± 0.04 ^{aB}	3.99 ± 0.07 ^{aB}
500	1.73 ± 0.01 ^{bA}	0.85 ± 0.02 ^{bA}	6.72 ± 0.00 ^{bB}	6.13 ± 0.16 ^{bB}

Mean values in columns with different lowercase letters are significantly different at $p < 0.05$, separately for germination and inactivation.

Mean values in rows with different capital letters are significantly different at $p < 0.05$, separately for germination and inactivation.

Table III
Germination and inactivation of *A. acidoterrestris* spores treated with 200 MPa at low and neutral pH in buffer solutions and in commercial apple juice (HP treatment at 50°C).

Time [min]	McIlvain buffer pH 4.0		McIlvain buffer pH 7.0		Commercial apple juice	
	Germination log [cfu/ml]	Inactivation log [cfu/ml]	Germination log [cfu/ml]	Inactivation log [cfu/ml]	Germination log [cfu/ml]	Inactivation log [cfu/ml]
5	1.84 ± 0.01 ^{AA}	0.18 ± 0.00 ^{AA}	1.67 ± 0.06 ^{AA}	0.07 ± 0.00 ^{AB}	2.65 ± 0.09 ^{AB}	0.34 ± 0.03 ^{AC}
10	2.31 ± 0.02 ^{BA}	0.75 ± 0.03 ^{BA}	2.12 ± 0.05 ^{BB}	0.14 ± 0.02 ^{BB}	2.99 ± 0.05 ^{ABC}	1.67 ± 0.09 ^{BC}
15	2.64 ± 0.04 ^{CA}	1.15 ± 0.04 ^{CA}	2.62 ± 0.01 ^{CA}	0.23 ± 0.02 ^{CB}	3.17 ± 0.08 ^{BB}	2.04 ± 0.16 ^{CC}
30	3.24 ± 0.00 ^{DA}	1.79 ± 0.00 ^{DA}	3.06 ± 0.00 ^{DA}	0.24 ± 0.01 ^{CB}	4.06 ± 0.30 ^{CB}	2.78 ± 0.03 ^{DC}

Mean values in columns with different lowercase letters are significantly different at $p < 0.05$, separately for germination and inactivation. Mean values in rows with different capital letters are significantly different at $p < 0.05$, separately for germination and inactivation.

Table IV
Germination and inactivation of *A. acidoterrestris* spores treated with oscillatory pressure 200 MPa at low and neutral pH in buffer solutions and in commercial apple juice (HP treatment at 50°C).

Number of cycle	McIlvain buffer pH 4.0		McIlvain buffer pH 7.0		Commercial apple juice	
	Germination log [cfu/ml]	Inactivation log [cfu/ml]	Germination log [cfu/ml]	Inactivation log [cfu/ml]	Germination log [cfu/ml]	Inactivation log [cfu/ml]
2	2.72 ± 0.06 ^{AA}	1.57 ± 0.05 ^{AA}	2.19 ± 0.09 ^{AB}	0.09 ± 0.05 ^{AB}	3.11 ± 0.18 ^{AA}	1.48 ± 0.27 ^{AA}
4	3.45 ± 0.04 ^{BA}	2.28 ± 0.05 ^{BA}	2.89 ± 0.03 ^{BB}	0.20 ± 0.15 ^{AB}	3.61 ± 0.06 ^{BC}	2.40 ± 0.03 ^{BA}
6	3.89 ± 0.10 ^{CA}	2.67 ± 0.01 ^{CA}	3.38 ± 0.03 ^{CB}	0.46 ± 0.04 ^{BB}	4.04 ± 0.01 ^{CA}	2.70 ± 0.04 ^{BA}

Mean values in columns with different lowercase letters are significantly different at $p < 0.05$, separately for germination and inactivation. Mean values in rows with different capital letters are significantly different at $p < 0.05$, separately for germination and inactivation.

30 min of continuous pressurization with 200 MPa at 50°C, 3.06 log of spores suspended in pH 7 buffer germinated, but only 0.24 log was inactivated. Germination in a pH 4.0 buffer and in commercial apple juice was higher (3.24 and 4.06 log respectively). In these conditions inactivation achieved 1.79 log in a pH 4.0 buffer and 2.78 log in apple juice. Part of the spore population still remained ungerminated.

The results achieved in this part of our study also show that the nutrients present in commercial apple juice can promote the germination of *A. acidoterrestris* spores during pressurization under moderate HP. The same phenomenon was observed by Vercammen *et al.* (2012), in tomato juice.

Many studies have demonstrated that the application of pressure cycling is more efficient than constant pressure treatment when the total exposure is equivalent (Hayakawa *et al.*, 1994; Furukawa *et al.*, 2000; Sokołowska *et al.*, 2012). Furukawa *et al.* (2000) concluded that hydrostatic pressure treatment initiated the germination of bacterial spores, and that repeated rapid decompression caused disruption, injury and inactivation of the germinated spores. Nguyen Thi Minh *et al.* (2010) suggest that after inducing germination of spores under the first pressure treatment, decompression between the pressure cycles favours the progression from first stage to second stage of the germinated spores. The second stage germinated spores are

then inactivated by subsequent pressure cycles, which would explain the greater spore destruction by pressure cycling. Recently this statement was disproven by Kong *et al.* (2014), who observed that spore germination stopped 5 to 10 min after the HP was released. Obtained in those study results suggest that an HP of 150 MPa for < 30 s is sufficient to fully activate spores' germinant receptors (GRs), which remain activated at 1 MPa but can deactivate at ambient pressure.

The results obtained (Table IV) showed that spores germinated after 2 cycles: 2.72 log in pH 4.0 buffer or 3.11 log in apple juice. Germination achieved 3.89 log in pH 4.0 buffer and 4.04 log in apple juice after 6 cycles. Inactivation was also effective and achieved 2.67 log and 2.70 log respectively. These results did not differ significantly ($p > 0.05$) for both media. Only after 4 cycles germination in apple juice was significantly higher than in pH 4.0 buffer.

Slightly, but significantly less ($p < 0.05$) germination was observed in pH 7.0 buffer but inactivation in these conditions was very small. After 6 cycles in pH 7.0 buffer, 3.38 log of spores germinated and only 0.46 log were inactivated.

The application of pressure in the oscillatory mode gave a slightly but significantly better effect on the germination and inactivation of *A. acidoterrestris* spores compared to a continuous process in buffers. In apple juice, the differences were not significant.

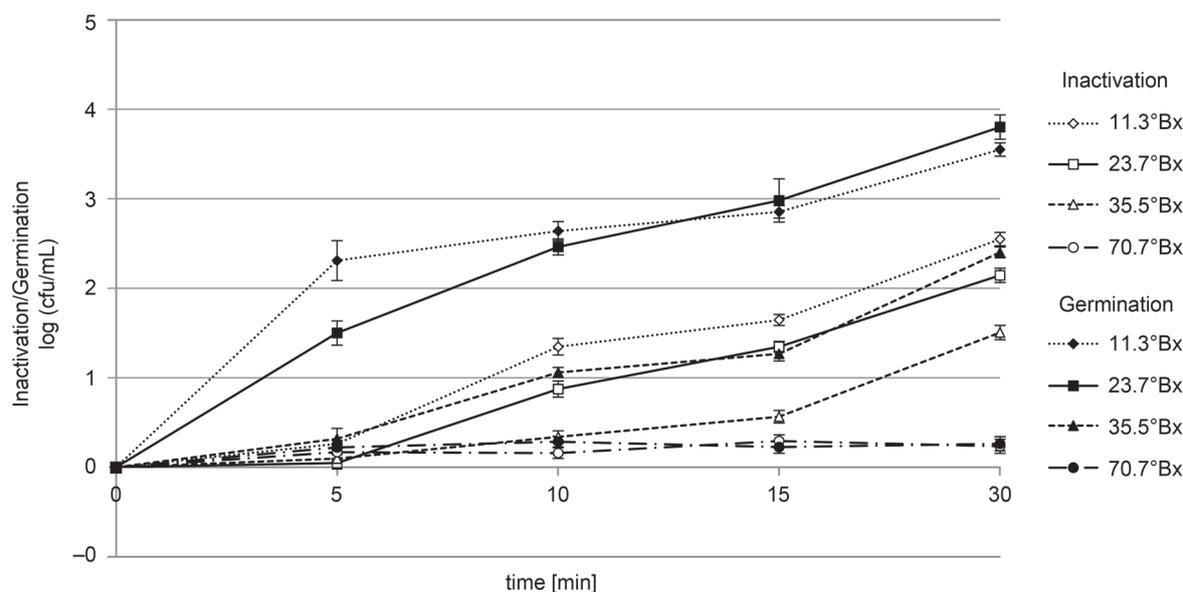


Fig. 2. Germination and inactivation of *A. acidoterrestris* spores treated with 200 MPa in apple juice with various soluble solids content (HP treatment at 50°C).

Inactivation was also low, compared to the results obtained in our previous study (Sokołowska *et al.*, 2012). In those experiments *A. acidoterrestris* TO-29/4/02 strain was used and inactivation achieved 5.0 log after 6 cycles at 50°C with 200 MPa, while 2.8 log inactivation was achieved after 30 min of continuous pressurization.

These different results may indicate large variations in the germination process under pressure, among *A. acidoterrestris* strains.

Influence of soluble solids content in apple juice on the germination and inactivation of *A. acidoterrestris* spores during pressurization. In this study pressure of 200 MPa at 50°C was used to investigate the influence of soluble solids content in apple juice on the germination of *A. acidoterrestris* spores.

As we expected after previous study (Sokołowska *et al.*, 2013) The baroprotective effect of an increase in the solute concentration in apple juice on *A. acidoterrestris* spores during high pressure processing was observed (Fig. 2). During 30 min pressurization of spores in concentrated apple juice (70.7°Bx), there was no significant germination and inactivation ($p > 0.05$). However, in juices with a soluble solids content of 35.7, 23.6 and 11.2°Bx, the spore germination was 2.40, 3.80 and 3.55 log after 30 min. In the same conditions inactivation was 1.51 log, 2.14 and 2.55 log, respectively. The results obtained demonstrate that the effect of high pressure combined with heat, against *A. acidoterrestris* spores, was highly dependent on the concentration of apple juice.

Similar results for the inactivation of spores were obtained by Lee *et al.* (2006). In the case of apple juice concentrate (70°Bx), treatment with high pressure (207, 414 and 621 MPa) at four different temperatures (22, 45,

71 and 90°C) showed no inactivating effect against the spores of *A. acidoterrestris* after 10 min of treatment. In diluted apple juice (17.5°Bx) *A. acidoterrestris* spore reductions was more than 5 log after 10 min at higher temperatures (71 and 90°C).

Increasing spore resistance with a greater soluble solids content may be explained by the lower a_w as well as by the protective effect of sugars. At low a_w germination may be incomplete as a result of water deficiency (Black *et al.*, 2007). A baroprotective effect of sugars was also reported for spores (Raso *et al.*, 1998).

Conclusions. The results of this study indicate that the treatment conditions, *i.e.* the level of pressure used and the temperature, time and mode of pressure application as well as, type and pH of the media and soluble solids content in apple juice considerably influenced the germination and inactivation of *A. acidoterrestris* spores. These factors should be kept in mind when designing moderate pressure treatments to assure the safety and stability of foods.

It was demonstrated that hydrostatic pressure treatment could induce germination and inactivation of *A. acidoterrestris* spores. A low pH favoured their germination and inactivation while a neutral pH suppressed inactivation. Increasing the process temperature strongly stimulated spore germination and inactivation. When the oscillatory mode was used the degree of germination and inactivation were slightly higher than at continuous mode. The degree of germination and inactivation was inversely proportional to the soluble solids content and was lowest in concentrated apple juice.

These results indicate that high inactivation of *A. acidoterrestris* spores might be possible by HP treatment

conducted at a moderately elevated temperature or followed by moderate heat treatment. This would allow better retention of the original properties, nutrients and bioactive components of the juices and make it possible to eliminate these spoilage bacteria.

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Autochthonous Gut Bacteria in Two Indian Air-breathing Fish, Climbing Perch (*Anabas testudineus*) and Walking Catfish (*Clarias batrachus*): Mode of Association, Identification and Enzyme Producing Ability

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Abstract

Scanning electron microscopy (SEM) was used to define the location of epithelium-associated bacteria in the gastrointestinal (GI) tract of two Indian air-breathing fish, the climbing perch (*Anabas testudineus*) and walking catfish (*Clarias batrachus*). The SEM examination revealed substantial numbers of rod shaped bacterial cells associated with the microvillus brush borders of enterocytes in proximal (PI) and distal regions (DI) of the GI tract of both the fish species. Ten (two each from the PI and DI of climbing perch and three each from the PI and DI of walking catfish) isolated bacterial strains were evaluated for extracellular protease, amylase and cellulase production quantitatively. All the bacterial strains exhibited high cellulolytic activity compared to amylolytic and proteolytic activities. Only two strains, CBH6 and CBH7, isolated from the DI of walking catfish exhibited high proteolytic activity. Maximum cellulase activity was exhibited by the strain, CBF2, isolated from the PI of climbing perch. Six most promising enzyme-producing adherent bacterial strains were identified by 16S rDNA gene sequence analysis. The strain ATH1 (isolated from climbing perch) showed high similarity to *Bacillus amyloliquefaciens* whereas, the remaining five strains (isolated from walking catfish) were most closely related to *Bacillus licheniformis*.

Key words: 16S rDNA, air-breathing fish, enzyme production identification, GI tract bacteria identification, SEM

Introduction

The microbial population within the gastrointestinal (GI) tract of fish is rather dense, with number of microorganisms much higher than those in the surrounding water, indicating that the digestive tract provides favourable ecological niches for these organisms (Ringø *et al.*, 1995). Some bacteria in the gastrointestinal tract are considered to be transient (allochthonous), whereas others exist as members of the established microbiota associated with the intestinal mucosa (autochthonous) (Ringø and Birkbeck, 1999). The autochthonous bacteria are responsible for enteric antagonism and colonization resistance, since they are associated closely with the intestinal epithelium, and form a barrier, serving as the first defense to limit direct attachment or interaction of fish pathogenic bacteria to the gut mucosa (Ringø *et al.*, 2007). Bacteria are the main constituents of the gut microbiota in fish (Spanggaard *et al.*, 2000; Pond *et al.*, 2006). The GI tract bacteria produce different types of extracellular enzymes that break down a wide range of substrates such as starch, cellulose,

chitin, protein, phytate and produce vitamin B12 (for review see Ray *et al.*, 2012).

To date, methods for studying the gut microbiota of fish, whether autochthonous or allochthonous, have been undertaken by homogenizing sections of gut and plating the homogenate onto a range of selective media. However, such methods detect only the microorganisms capable of growing on specific, selective media. A proportion of the bacterial population remains undetected. To define the presence of an autochthonous microbiota in fish, electron microscope examination might be a useful tool. This method has been used successfully in studies of the microbial ecology of the digestive tract of chickens, pigs, rodents and humans (Knutton *et al.*, 1987; Tannock, 1987). Although several publications have described the composition of intestinal microbiota in fish (Ringø *et al.*, 1995; Ringø and Birkbeck, 1999; Bairagi *et al.*, 2002; Mondal *et al.*, 2008; Ringø, 2008; Ray *et al.*, 2010; Dan and Ray, 2013), relatively few investigations have attempted to identify the location and mode of association of microorganisms in the GI tract using scanning electron microscopy

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(SEM) and/or transmission electron microscopy (TEM) (Hellberg and Bjerkaas, 2000; Lødemel *et al.*, 2001; Ringø *et al.*, 2001; Ringø *et al.*, 2002; Ringø *et al.*, 2003; Ghosh *et al.*, 2010). An in depth electron microscopical study on gut-associated bacteria is highly relevant as the digestive tract is a potential port of entry of pathogens. One remarkable feature of the indigenous (autochthonous) microbiota of fish gut is that they are affected by certain situations including stress, antibiotic administration and even small dietary changes. The stability of the gut flora is therefore, an extremely important factor in the natural resistance of fish to infections produced by bacterial pathogens in the GI tract. To the authors' knowledge, no information is available on the mode of association of GI tract bacteria in Indian air-breathing fish. Therefore, the aim of the present study was to detect autochthonous gut microbiota in the GI tract of two Indian air-breathing fish, the climbing perch, *Anabas testudineus* and walking catfish, *Clarias batrachus* by SEM. Furthermore, the presently reported study also investigated the protease, amylase and cellulase-producing capacity of selected bacterial strains in the proximal (PI) and distal intestine (DI) of the two air-breathing fish species through quantitative enzyme assay and identification of the most promising isolated gut-associated bacteria by 16S rDNA gene sequencing.

Experimental

Materials and Methods

Fish examined. Two species of adult Indian air-breathing fish, the climbing perch, *A. testudineus* and walking catfish, *C. batrachus* were selected for the present study. The fish were obtained from a local fish farm near Santiniketan, West Bengal, India (23°41'30" N latitude and 87°41'20" E longitude). The feeding habits (Jhingran, 1997), average live weight, relative intestinal length and average intestine weight of the fish species examined are presented in Table I. Five adult specimens

of each species were stocked separately in glass aquaria and starved for 48 hours in order to empty their alimentary tracts before dissection.

Tissue preparation for scanning electron microscopy. The selected portions of the GI tract were cut into two pieces, proximal and distal. The two segments were incised longitudinally. Each tissue segment was prepared step by step as follows: the tissue was fixed in cacodylate buffer (0.1 M) containing 2.5% glutaraldehyde for 1 h at 4°C, mucous was partially removed by washing the sample in heparinized saline (2 g heparin mixed in 20 ml of 0.67% NaCl), again washed with cacodylate buffer for four times (15 min each at 4°C) and kept in fresh buffer overnight at 4°C. Next day, the tissues were dehydrated through graded ethanol as follows: 50% (40 min), 70% (40 min), 90% (1 h) and absolute alcohol for 1 h. Thereafter, three consecutive changes were made in ethanol and amyl acetate in three different ratios 3:1, 2:2 and 1:3 (each for 30 min) and finally, tissues were kept in pure amyl acetate for 12 h. Critical point drying (CPD) was done in liquid nitrogen. The nitrogen was finally removed while in a supercritical state so that no gas-liquid interface is present within the sample during drying. The dry specimens were usually mounted on a specimen stub using an adhesive such as epoxy resin or electrically conductive double-sided adhesive tape followed by coating with gold particle in IB ion coater. Coated tissues were placed and observed with a Hitachi S530 Scanning Electron Microscope at an accelerating voltage of 20 kV. Images were taken and recorded digitally in Windows XP.

Isolation of gut associated bacterial flora. The GI tract of fish were dissected out aseptically inside the laminar air flow on an ice plate. It was cut into two parts, proximal (PI) and distal intestine (DI) and thoroughly rinsed three times with PBS (phosphate buffer saline, pH 7.2) to remove undigested food and allochthonous bacterial flora following the method described by Ringø (1993) before homogenization. After homogenization of the two parts separately, one ml of sample from each part were mixed in 0.9% of NaCl solution separately

Table I
Food habits, average live weight, average fish length, relative intestinal length and average intestine weight of the air-breathing fish species examined

Fish species	Feeding habit*	Average live weight (g) (SD)	Average fish length (cm) (SD)	Relative intestinal length	Average intestine weight (g) (SD)
<i>Anabas testudineus</i>	Insects, insect larvae, water fleas, smaller fish, vegetable debris etc.	98.4 (3.2)	16.4 (0.9)	0.43	7.2 (0.61)
<i>Clarias batrachus</i>	Insect larvae, shrimps, worms, small fish, organic debris etc. Carnivorous	138.2 (4.23)	18.3 (1.1)	0.50	9.2 (0.58)

* Jhingran (1997)

SD, Standard deviation ($n=5$)

Relative intestinal length = length of intestine (cm) / total length of fish (cm)

(Das and Tripathi, 1991) and serial dilution was done (Beveridge *et al.*, 1991). One hundred μ l of sample from each test tube was spread on tryptone-soya agar plate separately and incubated for 36 h at 34°C. The well separated colonies with distinct morphology were selected for pure culture. After pure culture bacteria were stored in TSA (tryptone soya agar) slant at 4°C.

Quantitative enzyme assay of the selected bacterial strains. For assessing the extracellular enzyme producing capacity of the bacterial strains, they were cultured in different broth media such as peptone-gelatin (g/l): beef extract 3 g, peptone 5 g and gelatin 4 g, starch (g/l): tryptone 2 g, KH_2PO_4 4 g, Na_2HPO_4 4 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, CaCl_2 0.001 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.004 g, starch 10 g and carboxymethylcellulose (CMC) broth (g/l): tryptone 2 g, KH_2PO_4 4 g, Na_2HPO_4 4 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, CaCl_2 0.001 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.004 g, CMC 10 for protease, amylase and cellulase, respectively. All the chemicals were supplied by HiMedia Laboratories Private Limited, Mumbai, India. For protease, amylase and cellulase enzyme production, the bacterial isolates were cultured in peptone-gelatin, starch and CMC medium, respectively in a shaker incubator. After incubation, the broth was centrifuged at 8,000 \times g and the supernatant was collected for enzyme assay. The assay of protease, amylase and cellulase were done according to Walter (1984), Bernfeld (1955), and Denison and Kohen (1977), respectively. Protein content of the supernatant was estimated by the method of Lowry *et al.* (1951). The specific enzyme activity was expressed as unit (U).

Identification of bacterial strains by 16S rDNA gene sequence analysis. Six most promising bacterial strains (one from *A. testudineus* and five from *C. batrachus*) were identified by 16S rRNA gene sequence analysis as described by Ringø *et al.* (2006). All sequences were aligned and analyzed using bioinformatics tool (Codon-code and Mega 4.0) for finding the closest homolog of the microbes using a combination of NCBI (National Centre for Biotechnology Information) GenBank and RDP (Ribosomal Database Project) database.

Results and Discussion

Mode of association. One of the criteria for testing autochthony of microorganisms in the GI tracts of fish is their association with the epithelial mucosa of the stomach, proximal or distal intestine (Ringø and Birkbeck, 1999). It has been suggested by several authors that electron microscopic (EM) examinations of the GI tract should be included as an important tool for investigating the microbial ecology of the gut ecosystem and determining the presence of autochthonous or allochthonous microbiota (for review, see Ray *et al.*, 2012). In the present study, scanning electron microscopy (SEM)

revealed sporadic colonization of autochthonous bacterial cells in the GI tract of both the fish species examined. Substantial association of rod-shaped bacterial cells on the tips and within the enterocyte microvilli in both PI and DI are demonstrated by SEM (Figs. 1–7). Figure 1 shows the association of rod-shaped bacteria in the apical aspects of the enterocytes in the PI of *C. batrachus*. Previous SEM and/or TEM elevations also demonstrated rod-shaped bacteria associated with the microvilli in the GI tract of rainbow trout, *Oncorhynchus mykiss* (Lesel and Pointel, 1979), Atlantic wolfish, *Anarhichas lupus* (Hellberg and Bjerkås, 2000), Arctic charr, *Salvelinus alpinus* (Ringø *et al.*, 2001) and rohu, *Labeo rohita* (Ghosh *et al.*, 2010). SEM investigation also revealed that the bacterial colonies attached in the intestinal fold were associated with mucous and the attachment of bacteria was clearly visible in the mucous removed intestinal folds (Figs. 2, 3 and 4). Similarly, attachment of autochthonous bacteria in GI tract of *A. testudineus* was also very clear (Figs. 5, 7). In the PI of *A. testudineus*, the luminal end of one bacterium is found protruding above the level of the microvilli (Fig. 6). In the DI of *A. testudineus*, numerous small pores are discernable (Fig. 8). These pores might be due to emptying mucous-producing goblet cells. Bacterial adhesion is a cell-surface interaction phenomenon which makes it ideal for examination by SEM (Knutton, 1995). Several factors are reported to influence adhesion and colonization of the microbiota within the GI tract. These are: (a) gastric acidity, (b) bile salts, (c) peristalsis, (d) digestive enzymes, (e) immune response and (f) indigenous bacteria and the antibacterial compounds produced by them (Ringø *et al.*, 2003). Ringø *et al.* (2007) reported that both bacterial cells and epithelial cells are negatively charged that prevent closer association of bacteria on the epithelial surface. SEM and classical microbiology have been used to investigate bacteria in different regions of the GI tract of rainbow trout, *O. mykiss* (Lesel and Pointel, 1979). In the presently reported study, in some cases, bacterial cells are seen to remain associated with the folds of the intestinal villi wrapped by mucous. This finding corroborates the observation made by Ghosh *et al.* (2010) in the GI tract of *L. rohita*. On the other hand, in the PI of *A. testudineus*, the bacteria are found to protrude above the level of microvilli as reported in Arctic charr, *S. alpinus* by Ringø *et al.* (2001). The mechanisms involved in adhesion of adherent bacteria in the GI tract seem to include adhesive factor(s), such as adhesion (Krovacek *et al.*, 1987), salinity and pH (Balebona *et al.*, 1995) and cell surface hydrophobicity (Parker and Munn, 1984; Bruno, 1988). In addition, receptor-specific interactions, such as pili-like structures, specific receptors on enterocytes such as sugar residues have been reported to be involved in mammalian models (Knutton, 1995).

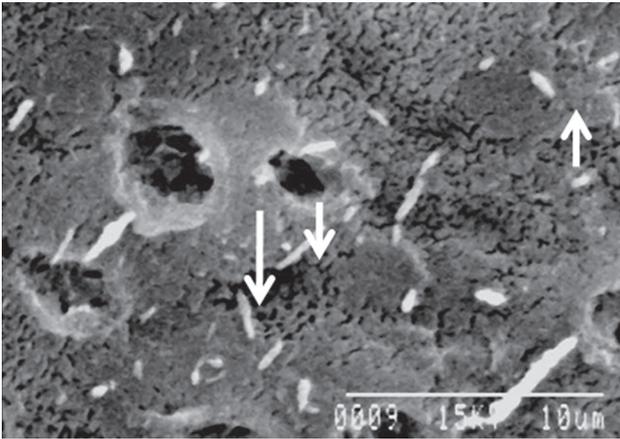


Fig. 1. Apical aspect of enterocytes in the PI showing association of rod-shaped bacteria (arrows) ($\times 5000$).

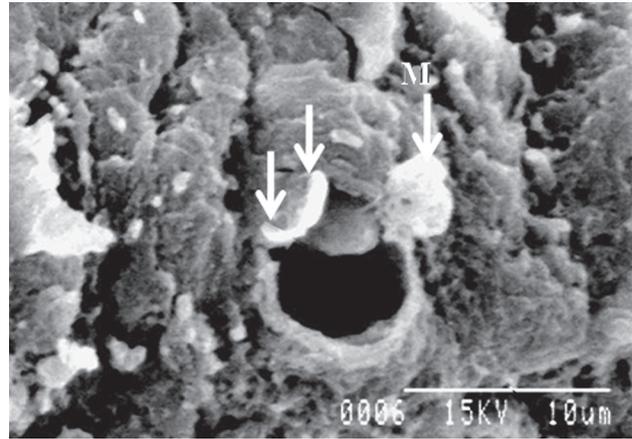


Fig. 2. Bacterial cells (arrows) are seen adjacent to the pore of goblet cell ($\times 4000$). M- indicate mucus.

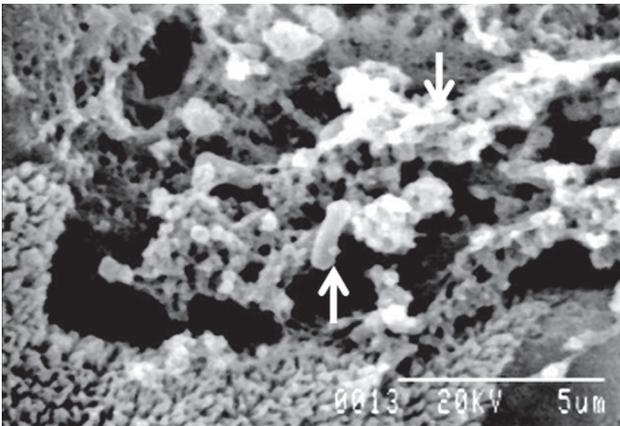


Fig. 3. Bacterial cells (arrows) are visible in close association with mucus ($\times 8000$).

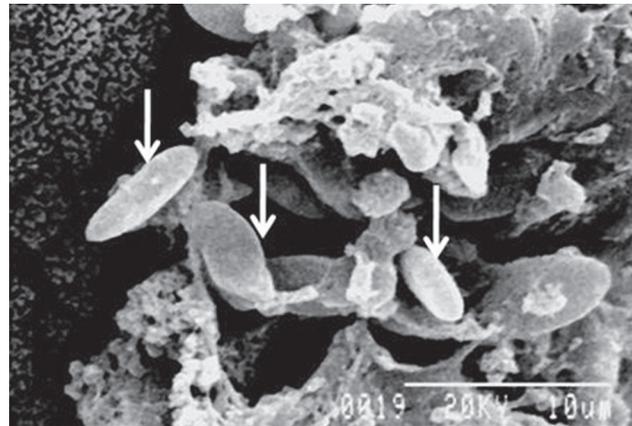


Fig. 4. Arrows indicate the presence of rod shaped bacteria which are comparatively larger in size ($\times 4000$).

Figs. 1–4. Scanning electron micrographs of PI and DI of *C. batrachus* showing bacterial cells associated with enterocytes.

Enzyme producing ability. Out of 21 strains isolated from the GI tract of the two fish species, 10 stains (2 each from the PI and DI of *A. testudineus* and 3 each from the PI and DI of *C. batrachus*) were the most promising with regard to enzyme producing ability. The results of quantitative enzyme activity exhibited by the bacterial isolates are presented in the Table II. All the bacterial strains exhibited high cellulase activity compared to amylase and protease activities. The strain CBF2 isolated from the PI of *C. batrachus* exhibited the highest cellulolytic activity (15.34 ± 0.11 U) followed by CBF4 and CBH5 (10.66 ± 0.24 U and 10.42 ± 0.073 U, respectively) isolated from the PI and DI, respectively of the same fish. The bacterial strain ATH1 isolated from DI of *A. testudineus* was also a good cellulase producer (1.92 ± 0.018 U). The presence of huge population of cellulolytic bacteria and their active role in extracellular cellulase production in fish has been confirmed in several investigations (Das and Tripathi, 1991; Saha and Ray, 1998; Bairagi *et al.*, 2002; Saha *et al.* 2006; Kar and Ghosh, 2008; Mondal *et al.*, 2008; 2010; Ray

et al., 2010). Considerable cellulolytic bacterial population has also been reported in the digestive tract in carnivorous murrel, *Channa punctatus* and stinging catfish, *Heteropneustes fossilis* (Kar and Ghosh, 2008; Banerjee *et al.*, 2013). Niederholzer and Hofer (1979) detected highest levels of cellulase activity in roach, *Rutilus rutilus*, and rudd, *Scardinius erythrophthalmus*, feeding on zooplankton and arthropods. Lindsay and Harris (1980) recorded moderate or high cellulase activity in gut contents of most “invertivores” feeding almost exclusively on invertebrates compared to omnivorous and piscivorous fish and concluded that cellulase activity in fishes is the direct result of ingestion of invertebrates containing cellulase or a cellulolytic microflora. Luczkovich and Stellwag (1993) also supported the hypothesis put forward by Lindsay and Harris (1980) and opined that cellulolytic bacteria present in the invertebrates consumed by pinfish, *Lagodon rhomboides* might have served as a source for the establishment and maintenance of the microbial flora. Our results corroborate these hypotheses and may explain

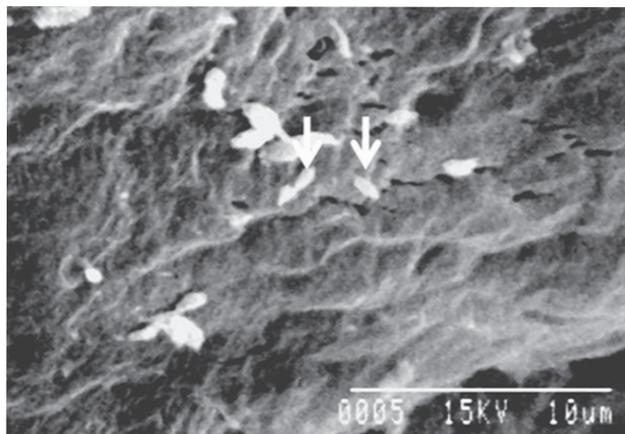


Fig. 5. Associated bacteria (arrows) are located at the apical brush border ($\times 5000$).

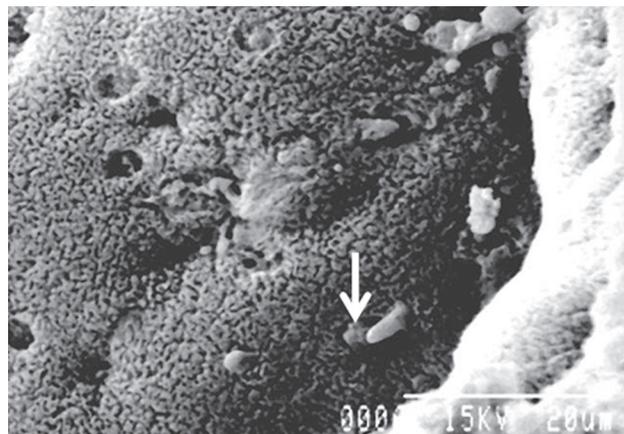


Fig. 6. The luminal end of one bacterium is seen protruding above the level of the microvilli (arrow) in the PI region ($\times 2000$).

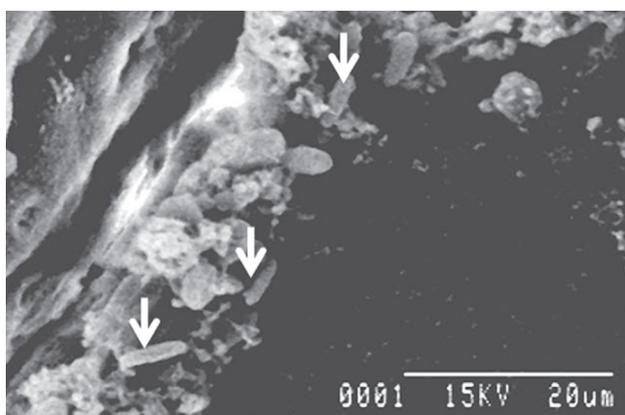


Fig. 7. Long rod-shaped bacteria (arrows) present on the epithelial surface of DI ($\times 2000$).

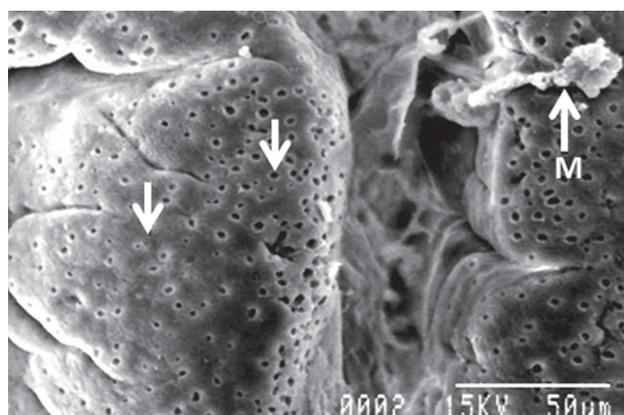


Fig. 8. SEM of DI showing numerous small pores (arrows) on the apical aspects of enterocytes ($\times 600$). *M. mucous*

Figs. 5–8. Scanning electron micrographs of the PI and DI of *A. testudineus* showing association of bacteria on the cell apices.

high cellulase activity in all the bacterial strains isolated from the GI tract of both the fish species studied which feed exclusively on invertebrates. In experimen-

tal conditions, cellulose was found to be poorly utilized by fish (Leary and Lovell, 1975; Anderson *et al.*, 1984; Shiau *et al.*, 1989; Hilton *et al.*, 1989). The results

Table II

Quantitative extracellular enzyme activities of the selected bacterial strains isolated from the GI tract of two air-breathing fish species

Fish species	Region	Strain designation	Protease (U) ¹	Amylase (U) ²	Cellulase (U) ³
<i>Anabas testudineus</i>	Proximal intestine	ATF4	0.51 (± 0.016)	0.02 (± 0.006)	0.71 (± 0.016)
		ATF5	0.69 (± 0.011)	0.46 (± 0.012)	1.80 (± 0.021)
	Distal intestine	ATH1	0.90 (± 0.019)	1.63 (± 0.13)	1.92 (± 0.018)
		ATH2	0.72 (± 0.02)	0.02 (± 0.004)	1.14 (± 0.011)
<i>Clarias batrachus</i>	Proximal intestine	CBF2	0.83 (± 0.026)	0.04 (± 0.01)	15.38 (± 0.11)
		CBF3	0.10 (± 0.015)	0.08 (± 0.02)	4.58 (± 0.14)
		CBF4	0.39 (± 0.032)	0.34 (± 0.041)	10.66 (± 0.24)
	Distal intestine	CBH5	0.16 (± 0.016)	0.14 (± 0.025)	10.42 (± 0.073)
		CBH6	1.13 (± 0.065)	0.16 (± 0.015)	4.58 (± 0.05)
		CBH7	1.82 (± 0.081)	0.06 (± 0.01)	6.51 (± 0.08)

Data are means \pm SE of three determinations.

¹ μ g of tyrosine liberated/mg protein/min; ² μ g of maltose liberated/mg protein/min; ³ μ g of glucose liberated/mg protein/min.

of those studies might raise questions regarding the role of cellulolytic bacteria in utilization of cellulose in fish. However, the presence of cellulolytic bacteria alone does not provide evidence that fish can utilize plant materials and/or cellulose. Symbiotic microbial enzymes, which hydrolyse cellulose, also generally require relatively long digestion times (Kristensen, 1972). So, in ruminant animals, a large fermentation/digestion chamber is present with a slow transfer rate to allow sufficient time and space for effective digestion. As fish do not possess a specialized chamber for bacterial fermentation, cellulose is sometimes poorly utilized by fish in spite of the presence of symbiotic cellulolytic bacteria. On the other hand, highest proteolytic activity was recorded in the bacterial strain CBH7 and CBH6 isolated from DI of *C. batrachus* (1.82 ± 0.081 U and 1.13 ± 0.065 U, respectively) followed by ATH1 isolated from DI of *A. testudineus* (0.9 ± 0.019 U). There are some published reports on protease production by fish GI tract bacteria (Skrodenytė-Arbačiauskienė, 2007; Ray *et al.*, 2010; Askarian *et al.*, 2012; Banerjee *et al.*, 2013). Bairagi *et al.* (2002), however, quantified the proteolytic activity in the bacterial strains isolated from nine freshwater teleosts. They recorded highest proteolytic activity in the bacterial strain TP3A, isolated from the gut of *Oreochromis mossambica*. While, Mondal *et al.* (2008) and Ray *et al.* (2010) also reported high proteolytic activity in the bacterial strains isolated from the DI of *Labeo calbasu* and three Indian major carps, *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*. Extracellular protease activity in these bacterial isolates indicates that there exists a definite source of microbial protease apart from the endogenous protease in fish GI tract which have contribution in host digestion process. In the present study, most of the isolated bacterial strains were not good amylase producers except ATH1 (1.63 ± 0.13 U), isolated from DI of *A. testudineus*. Kar and Ghosh (2008), however, reported amylase-producing bacteria in the digestive tracts of rohu (*L. rohita*) and murrel (*C. punctatus*). Mondal *et al.* (2008), however, could not detect amylolytic bacteria in the GI tract of carnivorous climbing perch, *A. testudineus*. They opined that amylase production by GI tract bacteria in herbivorous fish is much higher than that in carnivorous fish. Das and Tripathi (1991) reported high amylase activity in the gastrointestinal tract of grass carp (*Ctenopharyngodon idella*) which appeared to be the result of its omnivorous feeding habit. They are of opinion that there is a possibility of introduction of these enzyme producing microflora in fish GI tracts along with the food ingested, but, whether they form a persistent population in the gut is doubtful. Since, the amylolytic bacteria have been detected in fish GI tract after 48 h of starvation in the present study, it seems that some of the flora form a resistant population.

Table III
Identification of selected bacterial strains isolated from the GI tract of *Clarias batrachus* and *Anabas testudineus* with partial sequence of 16S rDNA genes referenced to accession no. in Gen Bank

Isolate	Closest relative (obtained from BLAST search)	Similarity (%)	Accession no.
ATH1	<i>Bacillus amyloliquefaciens</i>	99%	HG328254.1
CBF2	<i>Bacillus licheniformis</i>	97%	HQ005269.1
CBF4	<i>Bacillus licheniformis</i>	99%	HQ005269.1
CBH5	<i>Bacillus licheniformis</i>	98%	HQ005269.1
CBH6	<i>Bacillus licheniformis</i>	98%	HQ005269.1
CBH7	<i>Bacillus licheniformis</i>	100%	HQ005269.1

Identification of bacterial strains. All the strains isolated from the GI tract of both the fish species are Gram-positive, rod shaped aerobic bacteria with irregular configuration. Out of ten bacterial strains, six most promising ones (one from *A. testudineus* and five from *C. batrachus*) were identified by 16S rDNA sequence analysis (Table III). The 16S rDNA is the most conserved (least variable) gene in all cells. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. The bacterial strain ATH1 isolated from the DI of *A. testudineus* showed 99% similarity to *Bacillus amyloliquefaciens* (accession no. HG328254.1). *B. amyloliquefaciens* is a Gram-positive, rod shaped spore forming bacterium. This species of *Bacillus* has been isolated from different sources such as, fish gut (Ghosh *et al.*, 2010), brackishwater sediment (Cao *et al.*, 2011), plant (Chen *et al.*, 2009) and oil contaminated soil (Liu *et al.*, 2012). Due to high level of amylase production, it is considered to be a commercial producer in different industries (Deb *et al.*, 2013). The remaining five bacterial strains isolated from the GI tract of *C. batrachus* were close to *Bacillus licheniformis* (accession no. HQ005269.1). Different strains of *Bacillus* have been identified from the GI tract of fish (Ringø, 2008). In the present study, all the 6 identified strains belong to genus *Bacillus*. One of our strains (ATH1) showed 99% similarity to *B. amyloliquefaciens* (accession no. HG328254.1). *B. licheniformis* is a Gram-positive, mesophilic spore forming bacterium, previously reported in the GI tract of different freshwater fish species (Roy *et al.*, 2009; Mondal *et al.*, 2010; Banerjee *et al.*, 2013; Dan and Ray, 2014). *B. licheniformis* from different sources has been reported to be a valuable source for protease production (Asokan and Jayanthi, 2010; Degering *et al.*, 2012; Sangaralingam *et al.*, 2012; Banerjee *et al.*, 2013). Due to high protease producing capacity, *B. licheniformis* is used in different industries (Degering *et al.*, 2012).

Conclusions. The results of the present study provide evidence that autochthonous bacteria colonize in both the PI and DI of the two air-breathing fish species studied. The investigation further confirms that there is also a distinct microbial source of digestive enzymes (protease, amylase and cellulase) apart from the endogenous sources in fish GI tracts. Characterization and identification by 16S rDNA sequence analysis revealed that all the strains belong to the genus *Bacillus*. However, to present more reliable information on the gut microbiota in fish, several methods, such as random amplified polymorphic DNA, denaturing gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridization (FISH), and confocal microscopy have been used to identify and detect the microbial community in the GI tracts of fish. These techniques should be used in future studies while evaluating the bacterial community in the GI tract of Indian fishes.

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Physiology and Molecular Phylogeny of Bacteria Isolated from Alkaline Distillery Lime

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Abstract

This paper presents the results of the research on the number, taxonomic composition, and biochemical properties of bacterial strains isolated from the alkaline Solvay distillery lime, deposited at the repository in Janikowo (central Poland). Fifteen strains out of 17 were facultative alkaliphiles and moderate halophiles, and two were alkalitolerants and moderate halophiles. The number of aerobic bacteria cultured in alkaline lime was approximately 10^5 CFU ml⁻¹, and the total number of bacteria was 10^7 cells g⁻¹. According to 16S rRNA gene sequence analysis, nine strains belonged to the genus *Bacillus*, six to the genus *Halomonas*, one to the genus *Planococcus*, and one to the genus *Microcella*. Strains that hydrolyse starch and protein were the most numerous. Esterase (C4) and esterase lipase (C8) were detected in the majority of bacterial strains. Twelve strains exhibited α -glucosidase activity and nine, naphthol-AS-BI-phosphohydrolase activity. The present study proves that alkaliphilic bacteria of this type may constitute a source of potentially useful extremozymes.

Key words: *Bacillus* sp., *Halomonas* sp., alkaline solvay distillery lime, alkaliphiles, halophiles

Introduction

Extreme environments constitute a habitat for microorganisms with unique biochemical properties, useful for the industries, medicine, and environmental protection. In recent years the knowledge of extremophilic microorganisms which populate ecosystems generally considered unusual, has improved dramatically (Quazi, 2013). The main reason for the extensive research on extremophiles is the biotechnological potential of their cells and metabolites. These microorganisms are source of several biotechnologically relevant enzymes and biomolecules, e.g. cellulases, amylases, and ectoine (Jones *et al.*, 1998; Trotsenko and Khmelenina, 2002; Grant and Heaphy, 2010).

Microorganisms populating highly alkaline environments constitute a diversified group and can be divided into alkaliphiles and alkalitolerants. Alkaliphiles are capable of living in the environment with a pH of 10 and higher, thriving, however, in the environment with a pH of around 9 (their optimum pH), but incapable of

surviving in the environment with a pH of 7 or lower. On the other hand, alkalitolerants are capable of living in the environment with a pH higher than 10, although they thrive in environments with pH of 7 (their optimum pH is neutral) (Krulwich, 1986).

Alkaliphiles are found in natural alkaline environments such as saline soda lakes and ponds of East Africa (Foti *et al.*, 2006), Europe (Gerasimenko *et al.*, 2003), Asia (Antony *et al.*, 2013), Ca²⁺ alkaline springs of Near East, and soda deserts of North America (Duckworth *et al.*, 1996). Marine environments contain many alkalitolerant bacteria and usually a smaller number of alkaliphiles (Ntougias *et al.*, 2006; Tamura *et al.*, 2013). Alkaliphiles have also been isolated from a number of alkaline wastes, by-products from food processing, e.g. potato processing plant effluents (Collins *et al.*, 1983), olive and maize processing wastewater (Ntougias *et al.*, 2006; Sanchez-Gonzalez *et al.*, 2011).

Although alkaliphiles have been thoroughly studied, no information has been found about their isolation from a lime sludge, a by-product in Solvay soda process.

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Alkaline distillery lime deposited in sludge ponds on the premises of the manufacturing plant in Inowrocław and Janikowo (central Poland) is a potential habitat of alkaliphilic and alkalihalotolerant microorganisms due to its highly alkaline character (pH from 9.0 to 11.4) and increased salt concentration (up to 9% in dry matter). The main component of the alkaline lime is calcium carbonate (above 90%) and the remaining include amorphous calcium hydroxide (6%), silicon dioxide, magnesium, aluminum and chlorides (Ziółkowska *et al.*, 2013). Owing to strong alkaline properties, distillery lime increases the pH of aqueous solutions up to 11–12 (Jeliński *et al.*, 2011).

The aim of this research was to determine the abundance, physiology and molecular phylogeny of extremophilic microbiota originating from alkaline distillery lime.

Experimental

Materials and Methods

Study area. Alkaline distillery lime has been deposited in sludge ponds in Janikowo (central Poland, Kuyavia) since 1957. The repository of alkaline distillery lime, composed of several ponds separated by causeways, covers an area of 200 ha and has the capacity of over 13,000,000 Mg. It rises up to 16 m above the ground level (Regional Inspectorate for Environmental Protection, 2013).

Sampling sites. Alkaline distillery lime was collected in September 2012 from three ponds of different salinity: sampling site S (slightly saline), M (moderately saline), H (highly saline). Samples were collected using a Total Groundcare soil sampler (Zarzecze, Poland) from the surface layer of the deposits (depth of 0–20 cm) from plots with an area of 25 m². Placed in sterile glass jars, they were transported to the laboratory in a portable ice bag at the temperature below 4°C. In the laboratory, the samples from a particular sampling site were mixed thoroughly and subsamples for subsequent microbial and physico-chemical tests were placed in sterile glass jars. The analyses started within 4 hours of sample collection.

Chemical analysis of distillery lime. Gravimetric moisture of alkaline lime was determined with the method of mass loss in drying (Radojevic and Bashkin, 2006). Before the analysis, solid and dried samples were hammered to reduce agglomeration, then disintegrated and homogenized in a roller mill. The pH values of the samples were measured in an aqueous suspension using a deposit: solution ratio of 1 : 1 (w/w). Elmetron CPC-501 pH meter (Zabrze, Poland) was used for pH determination in the aqueous phase. The concentration of chloride ions was determined by argentometric

titration with silver nitrate (Radojevic and Bashkin, 2006). Total organic carbon (TOC) was measured in TOC 5000 analyzer combined with the SSM-5000A module (Shimadzu, Kyoto, Japan). TOC was calculated by subtracting inorganic carbon concentration (IC) from total carbon concentration (TC). Organic nitrogen concentration was calculated by subtracting ammonia nitrogen from total Kjeldahl nitrogen (TKN). TKN was determined in a Turbotherm system (Gerhardt, Königswinter, Germany). Ammonia nitrogen concentration was measured using Nessler method (Spectroquant Merck SQ 118, Darmstadt, Germany), after distillation in Gerhardt Vapodest 20 system (Gerhardt, Königswinter, Germany). Nitrate concentration was determined using spectrophotometric method (Spectroquant Merck SQ 118, Darmstadt, Germany) after extracting nitrate from the sample using 2.0 M KCl. Total phosphorus content was measured with the spectrophotometric molybdenum blue method (Spectroquant Merck SQ 118, Darmstadt, Germany) after sample digestion with aqua regia.

Isolation and enumeration of bacteria. To isolate bacterial populations from alkaline lime, 10 g from the three mixed samples was added into 90 ml of NaCl solution (concentration corresponding to the chloride content in a given sample). The mixtures were blended thoroughly, tenfold serial dilutions were prepared and 0.1 ml of each suspension was spread onto three replicate plates with the appropriate culture media.

Extracts from the alkaline lime (100 g dissolved in 1 l of distilled water, mixed for 20 min, filtered and adjusted to pH 7 and 11 using the appropriate buffer solutions according to Ntougias *et al.* (2006), were solidified with agar and used to estimate the number of cultivable bacteria. All plates were incubated up to 7 days at 25°C. The number of grown colonies was expressed as colony forming units (CFU) per gram (dry weight) of alkaline lime. To prepare bacterial strain collection intended for further physiological and taxonomy studies, single colonies appearing on isolation plates were subcultured in a medium containing (g l⁻¹) glucose 5, yeast extract 1, MgSO₄ 0.01, and NaCl 30, supplied with appropriate buffer system. The isolation was based on the following characteristics of bacterial colonies: color, size, shape, and prevalence on the medium.

The total number of bacteria in alkaline lime was determined by acridine orange direct count (AODC) technique after Hobbie *et al.* (1977). For this purpose, 1 g of alkaline lime (fixed with formaldehyde – 3.8% v/v final concentration, sterilized with MF-Millipore Membrane Filters, 0.22 µm) was placed in glass tubes containing 9 ml of filter-sterilized citric acid solution (final concentration 0.6 M), added to dissolve calcium carbonate in the samples. The suspensions were vortexed for 2 min. Triplicate subsamples were stained with

acridine orange (final concentration 0.01%) for 3 min (Hobbie *et al.*, 1977). At least 100 cells or all cells in 100 fields of view were counted under a microscope (Nikon Eclipse E200, Japan) for each slide.

Statistical analysis was conducted using Statistica 6.0 software and analysis of variance (ANOVA). The impact of two independent factors (pH and chloride concentration) on the number of cultivable bacteria and the total number of bacteria in alkaline distillery lime was compared. ANOVA was followed by Tukey's test (HSD).

Physiological studies. Cell morphology and the purity of cultures were checked routinely using a Nikon light microscope (Japan). All experiments were performed at 25°C, unless stated otherwise. Alkaliphilic/alkalitolerant strains were maintained and grown routinely on alkaline media (Ntougias *et al.*, 2006), composed of the nutritional base described above, 30 g l⁻¹ NaCl, 0.1 mM MgSO₄ and a buffer (Na₂CO₃-K₂HPO₄), at pH 10.

Bacterial tolerance to salt was investigated in media containing the nutritional base and 0.1 mM MgSO₄ with different NaCl concentrations (0, 30, 50, 80, 100, 150, 200 and 250 g l⁻¹ NaCl). Bacterial growth was measured as optical density at 600 nm after 7 days.

Nutrient media containing specific substrates (sodium caseinate, olive oil, starch, carboxymethyl cellulose), all at a concentration of 0.5% (w/v), 0.1 mM MgSO₄ and 0.05 g l⁻¹ yeast extract were used to evaluate the organic substrate utilization by the isolates. Frazier reagent (HgCl₂ 12 g, distilled water, 80 ml, concentrated HCl, 20 ml) was used to detect protein hydrolysis, Rhodamine B (1 mg ml⁻¹, Sigma, added to the medium after sterilization, followed by incubation and UV irradiation) to evaluate fat hydrolysis, Gram iodine to assess starch hydrolysis, and 0.1% Congo red and 1 M NaCl to determine cellulose hydrolysis. Alkaliphilic/alkalitolerant strains were investigated at pH 10 and 3% NaCl. The medium containing NaCl (pH 10) was used to investigate the growth temperature range (15, 20, 30, 40, 50°C). Anaerobic growth was tested using the BioMerieux GENbox anaerobic system at 25°C for 7 days. The ability of individual isolates to synthesize constitutive enzymes was studied using API ZYM system (BioMerieux, France).

Identification of bacterial strains based on the 16S rRNA gene. Total genomic DNA was extracted from the strains using the G-Spin Total DNA Extraction Kit (iNtRON Biotechnology, Sungnam, Korea) following the instructions given by the manufacturer. The 16S rRNA gene was amplified by PCR in a reaction mixture containing 1 U DreamTaq DNA polymerase (Thermo Scientific, Waltham, MA, USA), 0.2 mM dNTP mixture (Thermo Scientific), 1 × DreamTaq buffer (Thermo Scientific), 0.325 μM of primers 27F 5'-AGA GTT TGA TCM TGG CTC AG-3', (Lane, 1991) and 1492R 5'-TAC GGY TAC CTT GTT ACG ACT T-3' (Polz and Cavanaugh, 1998), 10 μg BSA (Thermo Scientific) and 1 μL genomic DNA in a total volume of 25 μL. Thermal profile consisted of an initial denaturation at 98°C for 5 min, 32 cycles of denaturation at 94°C for 0.5 min, annealing at 48°C for 0.5 min and extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR amplicons were checked under UV excitation on a 1 w/v% agarose gel stained with GR Safe DNA Stain (Innovita, Gaithersburg, MD, USA). PCR product purification, sequencing reaction with primer 27F and capillary electrophoresis were performed by the LGC Genomics GmbH (Berlin, Germany). Manual correction of automatic base calling on chromatograms was carried out using the Chromas software v 1.45 (Technelysium, Brisbane, QLD, Australia). Nucleotide sequences obtained in this study were submitted to GenBank under the accession numbers KJ870238-KJ870254.

Closest related species were identified with the EzTaxon online tool (Kim *et al.*, 2012). Sequence alignment with sequences retrieved from GenBank was performed with the SINA Aligner (Pruesse *et al.*, 2012). Phylogenetic analysis including the search for the best-fit model was conducted with the MEGA 6.0 software (Tamura *et al.*, 2013).

Results

All three types of distillery lime had alkaline pH (ranging from 10.2 to 11.3), measured in a water suspension (Table I). Chloride content ranged from 2.88%

Table I
Chemical properties of alkaline distillery lime

Sample	pH	Chlorides (%)	Water content (%)	Total organic carbon (%)	Organic nitrogen (%)	Nitrates (%)	Total phosphorous (%)
S	10.2±0.3	2.88±0.16	62.3±5.5	0.34±0.11	0.001	<LOD	0.006±0.0001
M	10.5±0.2	4.57±0.33	42.3±3.7	nm	nm	nm	nm
H	11.3±0.4	6.40±0.46	45.2±4.1	0.32±0.09	0.001	<LOD	0.008±0.001

LOD – limit of detection, nm – not measured

Table II
Abundance of bacteria in alkaline distillery lime

Sample	Cultivable bacteria (CFU · 10 ⁵ g dry mass)		Total number of bacteria (cells · 10 ⁷ g dry mass)
	pH=7	pH=10	
S	14.22 ± 1.19	2.74 ± 1.07	4.39 ± 1.72
M	4.59 ± 0.36	1.47 ± 0.11	18.29 ± 0.89
H	2.16 ± 0.37	1.06 ± 0.21	2.16 ± 0.93

to 6.40% and the water content ranged from 42.3% to 62.3%. The samples contained low amount of nutrients because total organic carbon content was low (only 0.34%). The organic nitrogen and total phosphorous contents were 0.001% and ~0.007%, respectively. The nitrate concentration was below the detection limit.

Two-way analysis of variance showed that both the number of cultivable microorganisms and the total number of bacteria in alkaline lime depended on its pH and chloride concentration ($p < 0.05$).

The results indicate that bacterial number at pH 7 ranged from 2.16 to ± 0.37 CFU · 10⁵ g⁻¹_{dw} in highly saline samples and from 14.22 to ± 1.16 CFU · 10⁵ g⁻¹_{dw} in slightly saline samples (Table II); at pH 11 these values ranged from 1.06 ± 0.21 CFU · 10⁵ g⁻¹_{dw} in highly saline samples and from 2.74 to ± 1.07 CFU · 10⁵ g⁻¹_{dw} in slightly saline samples.

Nine strains were isolated from the highly saline samples (marked H, strains H5-H13), four from the

moderately saline samples (marked M, strains M1-M4), and four from the slightly saline samples (marked S, strains S14-S17).

The total number of bacteria determined by direct cell count ranged from 2.16 ± 0.93 cells · 10⁷ g⁻¹_{dw} in the highly saline samples to 18.29 ± 0.89 cells · 10⁷ g⁻¹_{dw} in the moderately saline samples (Table III).

The majority of the bacterial isolates stained Gram-positive. Strains M2, M3, M4, H12, S14 and S15 were Gram-negative, which corresponds to the results of the molecular taxonomic identification. Strains M1, H6-H10 and H13 formed endospores. All strains were strictly aerobic (except strain S16) and oxidase-positive.

Physiological characteristics of bacteria isolated from alkaline distillery lime are presented in Table III. Nine bacterial strains (M1- M3, H5, H7, H8, H10, H11, H13) were able to grow at pH 7–11, and six (M4, H9, H12, S15-S17) at pH 6–11. Efficient growth of most strains was observed at the highest tested pH values and their optimal growth at pH 9–10 (facultative alkaliphiles). Only two strains (H6 and S14) had optimum pH of 7, but showed tolerance of pH values of up to 11 (alkalitolerant bacteria).

Ten isolates tolerated up to 200 g l⁻¹ NaCl, seven tolerated concentrations of up to 150 g l⁻¹ NaCl. Only one bacterial strain (S16) had low salt tolerance (up to 50 g l⁻¹ NaCl). Optimum salt concentration for growth of all the isolates were between 30 and 50 g l⁻¹ NaCl therefore they can be regarded as moderate halophiles.

Table III
Physiological characteristics of isolates from alkaline distillery lime

Isolate	pH range	NaCl (%, w/v)	Temperature (°C)	Degradation of organic compounds zone of hydrolysis (mm)			
				Starch	Protein	Lipids	Cellulose
M1	^a 7–11 (9)	0–20 (5)	^a 5–40 (30)	+15	+31	–	–
M2	6–11 (7)	0–20 (5)	5–40 (30)	–	–	–	–
M3	7–11 (9)	3–15 (5)	5–40 (20)	–	–	–	–
M4	6–11 (9)	0–20 (5)	10–40 (30)	–	–	–	–
H5	7–11 (9)	0–20 (3)	5–50 (30)	+20	+35	+35	–
H6	^a 7–11 (9)	0–15 (3)	5–40 (20)	+12	–	–	–
H7	7–11 (11)	0–20 (5)	5–40 ^a (20)	+12	+37	+33	–
H8	^a 7–11 (9)	0–15 (3)	5–40 (30)	+6	+/-	–	–
H9	^a 7–11 (10)	3–15 (3)	5–40 (20)	+18	+51	–	–
H10	^a 7–11 (10)	0–20 (3)	5–40 (20)	–	–	–	–
H11	7–11 (9)	0–20 (5)	5–40 ^a (30)	+16	+40	+30	–
H12	7–11 (7)	0–20 (5)	^a 5–40 (20)	+/-	–	–	–
H13	^a 7–11 (9)	0–15 (3)	5–40 (30)	+18	–	–	–
S14	6–11 (7)	0–15 (5)	10–40 (20)	+14	–	–	–
S15	6–11 (9)	0–20 (3)	5–40 (30)	–	–	–	–
S16	6–11 (10)	0–5 (0)	20–30 (30)	–	–	+4	–
S17	6–11 (9)	0–15 (3)	10–40 (30)	–	+25	–	–

^a – marginal growth

Table IV
Constitutive enzyme production

Enzyme	Isolate																
	M1	M2	M3	M4	H5	H6	H7	H8	H9	H10	H11	H12	H13	S14	S15	S16	S17
Alkaline phosphatase	-	-	+	+	-	-	-	+	-	+	-	+	-	+	-	+	-
Acid phosphatase	-	-	+	-	-	-	-	+	-	+	+	+	+	-	-	-	-
Esterase (C4)	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Esterase Lipase (C8)	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Lipase (C14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Leucine arylamidase	+	-	+	+	-	-	-	-	+	-	+	+	+	+	-	+	-
Valine arylamidase	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Cystine arylamidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trypsin	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α -chymotrypsin	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Naphtol-AS-BI-phosphohydrolase	+	+	+	-	-	+	-	+	+	-	+	-	+	-	-	+	-
α -galactosidase	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
β -galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β -glucuronidase	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
α -glucosidase	+	-	-	+	+	+	-	-	+	+	+	+	+	+	-	+	+
β -glucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
N-acetyl- β -glucosaminidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
α -mannosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α -fucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The optimum temperature for growth of the studied isolates (mesophiles) ranged from 20 to 30°C. However, most strains (except M4, S14, S16 and S17) could grow at 5°C.

Strains capable of hydrolyzing starch were the most numerous. All strains isolated from highly saline lime (H5-H13, except H10), plus strains M1 and S14 hydrolyzed this polysaccharide. Seven bacterial strains (M1, H5, H7, H9, H11, S17, H18) hydrolyzed protein, four (H5, H7, H11, S16) hydrolyzed fat while none hydrolyzed cellulose.

The results of the APIZYM tests (Table IV) confirm the activity of 15 enzymes out of 19 which were tested. Cystine arylamidase, β -galactosidase, α -mannosidase, and α -fucosidase were not detected. Esterase (C4) and esterase lipase (C8) were detected in all bacterial strains except H8.

Twelve strains exhibited α -glucosidase activity (M1, M4, H5, H6, H9-H13, S14, S16, S17). Nine strains exhibited naphtol-AS-BI-phosphohydrolase activity (M1-M3, H6, H8, H9, H11, H13, S16) and leucine arylamidase activity (M1, M3, M4, H9, H11-H13, S14, S16). Alkaline phosphatase was detected in seven strains (M3, M4, H8, H10, H12, S14, S16) and acid phosphatase – in six strains (M3, H8, H10-H13). Lipase C14 (S16), valine arylamidase (H11), trypsin (M3), α -chymotrypsin (M3), α -galactosidase

(H11), β -glucuronidase (H6), β -glucosidase (S16) and N-acetyl- β -glucosaminidase (S16) were detected only in single bacterial strains.

Molecular phylogenetic identification of strains revealed low diversity of cultivable bacteria, which belonged to four genera only (Fig. 1). *Microcella*, *Planococcus*, and *Halomonas* strains were isolated from the slightly saline samples, while *Bacillus* and *Halomonas* strains were isolated from the moderately and highly saline samples. In general, nine strains belonged to the genus *Bacillus*, six to *Halomonas*, while *Microcella* sp. and *Planococcus* sp. were represented by single isolates. The predominance of *Bacillus* species was the most significant in the highly saline samples.

Discussion

Alkaliphiles are a class of extremophiles which coexist with neutrophiles and inhabit extreme environments (Horikoshi, 1991). The number of alkaliphilic microorganisms in neutral soil samples ranges from 10^2 to 10^5 CFU g^{-1} , which corresponds to between 1/10 and 1/100 of the population of neutrophilic microorganisms (Horikoshi, 1991). In our study the ratio was higher, ranging from 1/2 to 1/7. The number of culturable aerobic bacteria in alkaline lime was approximately 10^5 CFU ml^{-1} in the alkaline medium. Our results are

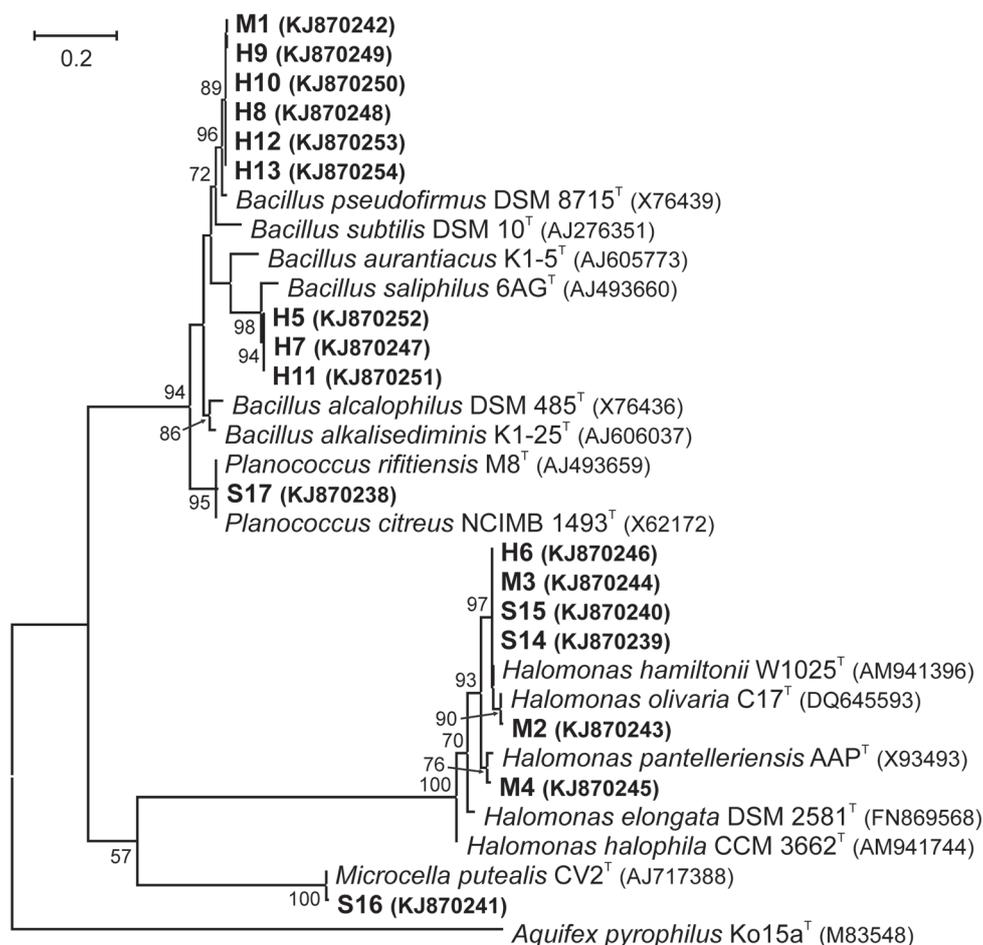


Fig. 1. Maximum likelihood phylogenetic tree based on the 16S rRNA gene including soda waste bacterial isolates and closely related type strains.

Explanations: The tree was reconstructed based on 781 nucleotide positions with the Tamura 3-parameter nucleotide substitution model. Bootstrap values lower than 50 were removed from the nodes. Sequences determined in this study appear in bold.

similar to those obtained by Ntougouias *et al.* (2006), who studied alkaline alpeorujó, a sludge-like by-product in olive oil extraction. However, the total number of heterotrophic bacteria in neutral medium was lower by two orders of magnitude in the alkaline lime. The difference may be caused by a considerably higher content of organic matter in alkaline alpeorujó (olives, olive leaves, and freshwater are a source of neutrophiles) compared to the nutrient-poor alkaline lime, consisting almost entirely of calcium carbonate.

The total number of bacteria in alkaline lime (approx. 10^7 cells g^{-1}) was higher than the total number of bacteria in carbonate rock cores collected from cave walls in the Bahamas (Schwabe *et al.*, 1997). On the other hand, it was lower compared to dolomites and soil, where it ranged from 10^8 to 10^9 cells g^{-1} (Ellis *et al.*, 2003; Su *et al.*, 2004; Maron *et al.*, 2006; Stres, 2007; Sheibani *et al.*, 2013).

The present study is the first attempt to investigate the distribution of bacteria in alkaline distillery lime. The results of the physicochemical analyses indicate that lime is a very harsh environment due to high pH

and low nutrient level. Depositing alkaline lime for over fifty years has promoted the selection of microorganisms capable of growing in a nutrient-poor, highly alkaline, and moderately saline environment. As a result, the majority of the bacterial strains isolated from the lime samples were facultative alkaliphiles and moderate halophiles.

Bacillus pseudofirmus, moderate halotolerant and obligate alkaliphile, first isolated from soil and animal manure (Nielsen *et al.*, 1995), was represented by six isolates (M1, H8, H9, H12, H13). These extremely halotolerant, facultatively alkaliphilic bacteria show marginal growth at pH 7 and optimum growth at the temperature of 20–30°C.

The closest relative of three isolates: H5, H7 and H11 was *Bacillus luteus*, first isolated from soil sample collected from Mandpam, Tamilnadu, India (Subhash *et al.*, 2014).

Planococcus rifietoensis, the closest relative of strain S17, is a halotolerant, alkaliphilic bacterium, first isolated from the algal mat formed at the sulfurous spring in Rifieto, Italy (Romano *et al.*, 1996).

Halomonas sp. are ubiquitous, moderately halophilic/halotolerant Gram-negative bacteria found in saline lakes, saline soils, marine environments, and solar salt extraction facilities. Several alkaliphilic species of *Halomonas* can live in soda lakes and alkaline soils (Máthé *et al.*, 2014). *Halomonas hamiltonii*, the closest relative of strains M3, H6, S14, and S16, is a halophilic, facultative alkaliphilic rod-shaped bacterium, isolated from patients' blood and dialysis machines in a nursing home (Kim *et al.*, 2010). *Halomonas olivaria*, the closest relative of bacterial strain M2 is a moderately halophilic, alkalitolerant bacterium, isolated from olive-processing effluents (Amouric *et al.*, 2014). *Halomonas pantelleriensis*, the closest relative of strain M4, is a haloalkaliphilic rod-shaped bacterium with optimum growth at pH 9.0, isolated from hard sands of the lake Venere in the Pantelleria island, Italy (Romano *et al.*, 2003).

Microbacteriaceae are widespread in natural and artificial environments such as soil, sewage, seawater and fresh water, plants, mushrooms, insects, and dairy products (Kageyama *et al.*, 2007) although they have been rarely isolated from alkaline environments. They include *Microcella putealis*, the closest relative of strain S16, a Gram-positive alkaliphilic bacterium isolated from a non-saline, highly alkaline groundwater (Tiago *et al.*, 2006).

The few available reports concerning microbial diversity of alkaline environments indicate the existence of many physiological groups (Duckworth *et al.*, 1996; Jones *et al.*, 1998; Horikoshi, 1999; Ntougias *et al.*, 2006; Felföldi *et al.*, 2009). The aerobic microbial population of these ecosystems contains organotrophic bacteria, including members of the *Bacillus* species, which are able to produce a wide range of enzymes used for recycling biopolymers: proteases, lipases, amylases, and cellulases. Strains capable of decomposing starch were the most numerous among the bacteria isolated from alkaline lime. Seventy percent of the isolated strains produced α -glucosidase, which breaks down starch and disaccharides to glucose. Bacterial amylases were extensively investigated because of their wide application in detergent, food, textile, and paper industries. Amylases with pH optimum of 9–12 have been identified in several alkaliphilic *Bacillus* spp. (Wang *et al.*, 2006; Fujinami *et al.*, 2010; Raval *et al.*, 2014).

In addition to amylolytic strains, the investigated alkaline lime contained bacteria capable of hydrolyzing proteins. More than half of the bacterial strains showed leucine arylamidase activity and one of these strains synthesized trypsin and α -chymotrypsin. Alkaline proteases secreted by both neutrophilic and alkalophilic bacteria seem interesting because they constitute a major source of proteolytic enzymes produced commercially (Horikoshi, 1999; Fujinami *et al.*, 2010). Alkaliphilic

proteases are widely applied in the detergent manufacturing (Wang *et al.*, 2006), leather tanning (Vijay Kumar *et al.*, 2011), food processing (Klomkdao *et al.*, 2012), and recovering silver from X-ray films (Shankar *et al.*, 2010). *Bacillus* species are main producers of many alkaline proteases, already isolated and described.

Bacteria which are able to synthesize lipase and decompose olive oil containing long chain fatty acids constituted the least numerous physiological group among the bacterial strains isolated from alkaline lime. Four isolates growing on agar plates with olive oil and Rhodamine B (indicator) hydrolyzed the substrate, releasing free fatty acids (producing an orange halo around the bacterial colonies). Surprisingly, the results of the APIZYM test revealed that only strain S16 showed (low) activity of lipase (C14). On the other hand all strains except one synthesized lipase/esterase (C8), which catalyze the hydrolysis of short chain fatty acids. Numerous applications of lipases include organic synthesis (Jiang *et al.*, 2013), hydrolysis of fats and oils (Bourlieu *et al.*, 2012), modification of fats (Gupta *et al.*, 2003), flavor enhancement in food processing (Chávez *et al.*, 2011), resolution of racemic mixtures, and chemical analyses (Sharma *et al.*, 2011).

As has been demonstrated in several studies, some alkaliphilic *Bacillus* spp. produce cellulases which can be used for improving the efficiency of detergents (Ito, 1997). However, bacterial strains isolated from alkaline lime were unable to decompose carboxymethyl cellulose (CMC).

The comparison of taxonomic identification of the investigated microorganisms with their physiological and ecological properties and the activity of identified enzymes indicates that they were highly variable within the members of the same species (e.g. H5, H7 and H11 or H6, M3, S14 and S15). On the other hand, protein and lipid hydrolysis were detected only in strains that belonged to the genus *Bacillus* (and also in a *Microcella* strain).

The pilot data presented shows that a viable alkaliphile community is present in the highly alkaline, distillery lime, a by-product in Solvay' soda process. The species composition of the microbial population strongly depends on pH values and salt concentration. Moreover, alkaline lime is a poor in nutrients, oligotrophic environment. Autochthonous microorganisms can survive in these unfavorable conditions owing to their ability to form spores. Therefore the dominant genus among cultivable bacteria is *Bacillus*. The surface layer of the lime may be colonized by microorganisms transferred from the surrounding saline soils and Lake Pakoskie, situated 500 m West of the repository ponds.

The obtained results confirm that the isolates display various enzymatic activity; they can decompose starch, protein and fat. Past experiences at such

environments has shown that it is extremely likely that alkaline adapted and tolerant microbes may be useful for man. The results of this study will provide foundation for further detailed research on extremozymes secreted by the isolates.

Applied in this research culture-based method are naturally biased in their evaluation of microbial genetic diversity by selecting a small (<1%), particular population of microorganisms present in a given environment. However the use of cultural enrichment techniques has its place for screening and isolating organisms for potential biotechnological application where industrial-scale growth in process systems, and hence amenability to growth in such systems, is required (Ritz, 2007).

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Inhibitory Effect of Newly-Synthesized Chalcones on Hemolytic Activity of Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Pathogenicity of methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with a broad spectrum of virulence factors, amongst which is α -hemolysin. The aim of this study was to investigate the effect of three newly-synthesized chalcones (1,3- Bis-(2-hydroxy-phenyl)-propenone, 3-(3-Hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone and 3-(4-Hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone) on α -hemolysin production of clinical isolates of MRSA. Subinhibitory concentrations of the tested compounds reduced hemolytic activity of MRSA strains, with almost complete abolishment of hemolysis at concentrations in the range of 1/2–1/4 x MIC (25–12.5 μ g/ml). In conclusion, newly-synthesized chalcones tested in this study showed potent inhibitory activity on α -hemolysin production of multiresistant and genetically diverse MRSA strains.

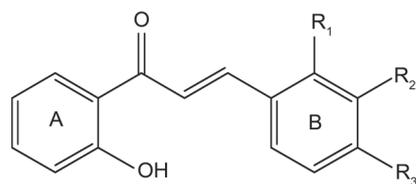
Key words: α -hemolysin, chalcones, MRSA

Pathogenicity of methicillin-resistant *Staphylococcus aureus* (MRSA) is directly associated with a broad spectrum of virulence factors, amongst which is α -hemolysin (*i.e.* HIIa or α -toxin). It is generally considered that α -hemolysin plays a central role in the pathogenesis of staphylococcal infections, especially in pulmonary infections caused by these bacteria (Bubeck Wardenburg *et al.*, 2007; Burlak *et al.*, 2007; Montgomery *et al.*, 2008). Approximately one half of staphylococcal necrotizing pneumonia cases affecting previously healthy adults and children are caused by community-associated MRSA strains (Ragle and Bubeck Wardenburg, 2009). Besides direct lysis of the pulmonary cells, α -hemolysin also activates alveolar macrophages or monocytes, induces massive polymorphonuclear leukocyte influx into lung parenchyma with subsequent degranulation and destruction of microvascular endothelium and adjacent tissues, and induces platelet-neutrophil co-aggregation leading to the host response to toxin that contribute to the severity of lung destruction (Parimon *et al.*, 2013). Such complex pathogenicity and occurrence of multiresistant strains

urge the development of antimicrobial compounds that selectively target virulence factors. In past years, several studies investigated the inhibitory activity of various compounds against extracellular virulence factors of *S. aureus* (Escaich, 2008; Cegelski *et al.*, 2008). Previously we have reported antimicrobial activity of three newly-synthesized chalcones against clinical isolates of MRSA, and inhibitory effect on the expression of virulence factors related to the early step of bacterial invasion-adherence (*i.e.* biofilm formation, glycocalyx production and adherence to human fibronectin) (Božić *et al.*, 2014). Therefore, the aim of this study was to investigate the effect of 1,3- Bis-(2-hydroxy-phenyl)-propenone (O-OH), 3-(3-Hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone (M-OH) and 3-(4-Hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone (P-OH) on α -hemolysin production of clinical isolates of multiresistant and genetically diverse strains of MRSA.

Antibacterial activity of chalcones was tested against 20 clinical isolates of MRSA isolated from blood (4), wound (6), sputum (3), endotracheal tube (2), abdominal drain (1), nose (1), skin (1), urine (1) and external

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Compound	R1	R2	R3
O-OH	- OH	- H	- H
M-OH	- H	- OH	- H
P-OH	- H	- H	- OH

Fig. 1. The chemical structures of the tested chalcones:

- 1,3- Bis-(2-hydroxy-phenyl)-propenone (O-OH)
 3-(3-hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone (M-OH)
 3-(4-hydroxy-phenyl)-1-(2-hydroxy-phenyl)-propenone (P-OH)

auditory canal (1) and one laboratory control strain of methicillin-resistant *S. aureus* ATCC 43300 (KWIK-STIK™, Microbiologics, USA). Identification of the isolates and methicillin resistance were determined by VITEK 2 test cards GP and AST-P580 (bioMérieux, France) and confirmed by PCR for *nuc* (Brakstad *et al.*, 1992) and *mecA* (Bignardi *et al.*, 1996) genes. Genotyping (SCC*mec*, *agr*, *pvl* and *spa* typing) of MRSA was performed according to previously described protocols (Boye *et al.*, 2007; Lina *et al.*, 2003; Harmsen *et al.*, 2003). The *spa* types were clustered into *spa* clonal complexes (CCs) using the algorithm based upon repeat pattern (BURP) with Ridom Staph-Type 1.4 software (<http://www.ridom.de>). The multiresistance profile of MRSA strains was determined by VITEK 2 test card AST-P580 and further supplemented with disc diffusion test according to CLSI guidelines (CLSI, 2007). Chalcones tested in this study (Fig. 1) were obtained from the Department of Pharmaceutical Chemistry, University of Belgrade-Faculty of Pharmacy, Belgrade,

Serbia. Compounds were prepared and characterized as previously described (Božić *et al.*, 2014) and their antimicrobial activity was determined by broth microdilution test according to CLSI guidelines (CLSI 2007). Hemolytic activities of MRSA culture supernatants were determined according to the method of Rowe and Welch (1994). The data obtained in this study were analyzed in SPSS statistical program (PASW statistics for Windows, Version 18.0, Chicago: SPSS Inc. USA) using methods of descriptive statistics, Chi square test and Mann-Whitney U test.

Clinical isolates of MRSA were genetically heterogeneous and expressed multiresistance phenotype (Table I). MRSA strains were classified into SCC*mec* type I (55.5%), II (5.0%), III (20.0%), IV (10.0%) and V (10.0%) and *agr* type I (35.0%), II (60.0%) and III (5.0%). Strains belonged to 10 *spa* types and were clustered into 5 *spa* CCs, with most frequent CC5 (55.0%) and CC8 (20.0%).

Tested chalcones exerted inhibitory activity against MRSA, with the order of potency of chalcones (average MIC \pm SD) as following: O-OH (MIC = 37.5 \pm 13.2 μ g/ml) > M-OH (MIC = 97.5 \pm 27.5 μ g/ml) > P-OH (MIC = 110.8 \pm 21.1 μ g/ml). The most significant dose-dependent inhibition of hemolysis was observed in MRSA supernatants cultivated with O-OH chalcone (Fig. 2). A 91.6–99.7% reduction of hemolysis was detected in all MRSA strains cultivated with $\frac{1}{2}$ \times MIC of O-OH. Hemolytic activity of MRSA strains cultivated with $\frac{1}{2}$ \times MIC (25.0 μ g/ml) and $\frac{1}{4}$ \times MIC (12.5 μ g/ml) of O-OH was in the range of 3.8–8.2% of the positive control ($p < 0.001$). Mild increase of the hemolytic activity was detected after cultivation with $\frac{1}{8}$ \times MIC (6.2 μ g/ml) up to 19% ($p < 0.01$), and $\frac{1}{16}$ \times MIC (3.1 μ g/ml) up to 34.6% ($p < 0.01$). The effect of M-OH

Table I
 Genotyping and resistance profiles of MRSA strains.

CC type	<i>spa</i> type	N° of strains	SCC <i>mec</i> type	<i>agr</i> type	PVL	Resistance profile (> 50% of strains)
CC5	<i>t041</i>	9	1	2	-	AM, GEN, KAN, TOB, STR, LIN, CLIN, ER, CLA, AZ, SPIRA, TET, DOX, CHL
	<i>t041</i>	1	1	1	-	AM, GEN, KAN, TOB, STR, LIN, CLIN, ER, CLA, AZ, SPIRA, CHL
	<i>t003</i>	1	2	2	-	KAN, TOB, LIN, CLIN, ER, CLA, AZ, SPIRA
CC8	<i>t030</i>	2	3	1	-	AM, GEN, KAN, TOB, STR, ER, CLA, AZ, TET, DOX, MIN, CHL
	<i>t030</i>	1	3	2	-	AM, GEN, KAN, TOB, STR, LIN, CLIN, ER, CLA, AZ, SPIRA, TET, DOX, MIN
	<i>t969</i>	1	3	1	-	AM, GEN, KAN, TOB, STR, ER, CLA, AZ, TET, DOX
CC45	<i>t015</i>	1	1	2	-	AM, GEN, KAN, TOB, STR, LIN, CLIN, ER, CLA, AZ, SPIRA, CHL
	<i>t583</i>	1	4	1	-	AM, GEN, KAN, TOB, STR, ER, CLA, AZ
CC80	<i>t044</i>	1	4	3	+	KAN, STR, TET
CC152	<i>t595</i>	1	5	1	+	GEN, KAN, TOB, LIN, TET
	<i>t6371</i>	1	5	1	-	GEN, KAN, TOB, ER, CLA, AZ

AM – amikacin, GEN – gentamicin, KAN – kanamycin, TOB – tobramycin, STR – streptomycin, LIN – lincomycin, CLIN – clindamycin, ER – erythromycin, CLA – clarithromycin, AZ – azithromycin, SPIRA – spiramycin, TET – tetracycline, DOX – doxycycline, MIN – minocycline, CHL – chloramphenicol.

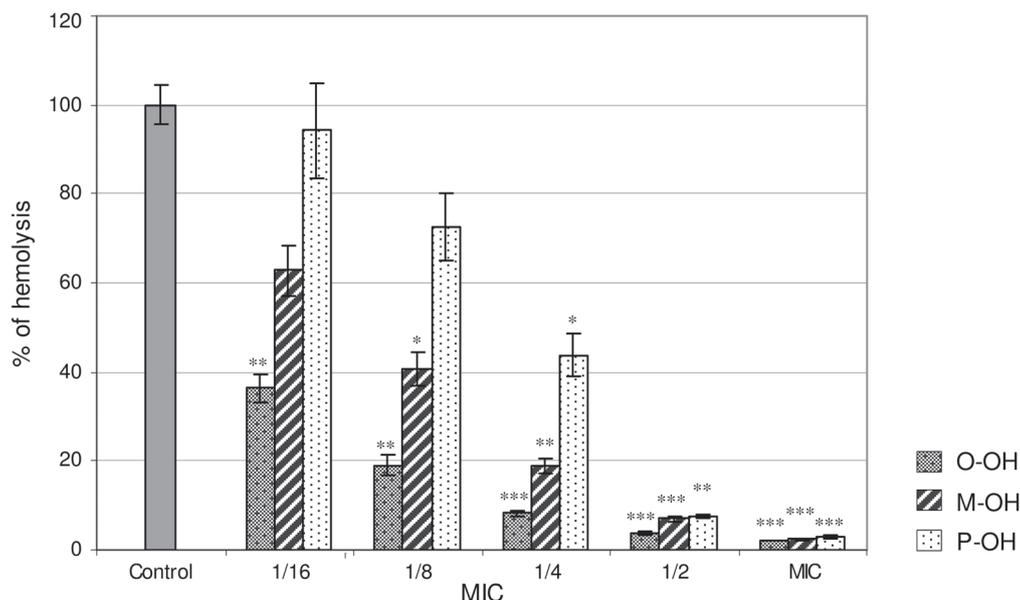


Fig. 2. The inhibitory effect of the O-OH, M-OH and P-OH chalcone on α -hemolysin production of MRSA strains. Results are present as mean percent of hemolysis \pm SD of 20 clinical isolates of MRSA and one control strain (MRSA ATCC 43300), compared to the positive control (*i.e.* hemolytic activity of supernatants of untreated MRSA strains presented as 100% hemolysis); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

and P-OH was tested in the two-fold higher concentrations than O-OH chalcone ($50.0\text{--}6.2\ \mu\text{g/ml}$), corresponding the same range of MIC's ($1/2 \times \text{MIC} - 1/16 \times \text{MIC}$). Hemolytic activity of MRSA strains cultivated with $1/2 \times \text{MIC}$ and $1/4 \times \text{MIC}$ of M-OH was in the range of 7.1–18.8% of the positive control ($p < 0.01$; $p < 0.001$), and cultivation with $1/8 \times \text{MIC}$ lead to the increase of the hemolysis up to 40.7% of the positive control ($p < 0.05$). The lowest applied concentration of M-OH ($1/16 \times \text{MIC}$, $6.2\ \mu\text{g/ml}$) did not reduce the hemolytic activity of MRSA ($p > 0.05$). Statistically significant inhibition of hemolytic activity of MRSA strains cultivated with P-OH chalcone occurred only in concentrations of $1/2 \times \text{MIC}$ (7.7%; $p < 0.01$) and $1/4 \times \text{MIC}$ (43.8%; $p < 0.05$) (Fig. 2). Chalcones themselves did not perform any hemolytic activity or induced hemolysis of rabbit erythrocytes in the same range of MIC's ($1/2 \times \text{MIC} - 1/16 \times \text{MIC}$; $p > 0.05$).

Antistaphylococcal activity of various chalcones is strongly correlated with their chemical characteristics. It is generally considered that lipophilicity of the ring A (Nowakowska, 2007) and presence of free hydroxyl group/s at various positions of the ring B are necessary for antistaphylococcal activity of these compounds (Sato *et al.*, 1996; Alcaraz *et al.*, 2000; Kromann *et al.*, 2004; Talia *et al.*, 2011). Three compounds investigated in our study carrying free hydroxyl group at various positions of the B ring exerted strong anti-MRSA activity, with 1,3-Bis-(2-hydroxy-phenyl)-propenone (O-OH) bearing the hydroxyl group at the position 2 of ring B as the most active one. These results are in accordance with the findings of other investigators (Alcaraz *et al.*, 2000;

Kromann *et al.*, 2004). Subinhibitory concentrations of the tested compounds also inhibited hemolytic activity of MRSA strains in a dose dependent manner, with almost complete abolishment of hemolysis at concentrations in the range of $1/2\text{--}1/4$ MIC ($25\text{--}12.5\ \mu\text{g/ml}$). Inhibition of hemolytic activity of α -hemolysin was dose dependent. Chalcone with free hydroxyl group at the position 2 of ring B was the most active one, since the lowest applied concentration of this compound ($1/16 \times \text{MIC}$, $3.1\ \mu\text{g/ml}$) exerted statistically significant inhibition of hemolysis. Higher concentrations of other tested compounds exerted similar inhibitory effect, with partial or complete recovery of hemolytic activity of α -hemolysin when applied in the lowest concentrations ($1/16 \times \text{MIC}$, $6.2\ \mu\text{g/ml}$). Previously it has been reported that subinhibitory concentrations of chalcones with similar chemical structure isolated from natural sources, like licochalcone A and E, inhibit α -hemolysin production (Qiu *et al.*, 2010a; Zhou *et al.*, 2012). Licochalcone A possess anti-MRSA activity in the range of MIC's from $2.0\text{--}8.0\ \mu\text{g/ml}$ (Fukai *et al.*, 2002; Qiu *et al.*, 2010a), and inhibitory effect on α -hemolysin production in the range of $1/2\text{--}1/8 \times \text{MIC}$ (Qiu *et al.*, 2010a). Licochalcone E was found to be the most active licochalcone against *S. aureus*, since the inhibitory effect was detected in the range of MIC's from $1.0\text{--}4.0\ \mu\text{g/ml}$, and inhibition of α -hemolysin production in the range of $1/4\text{--}1/16 \times \text{MIC}$ (Zhou *et al.*, 2012).

Precise mechanism of antimicrobial activity of chalcones and inhibition of α -hemolysin synthesis has not been clarified yet. Several different mechanisms could be involved, like direct enzyme inhibition, inhibition

of DNA or RNA synthesis (Mori *et al.*, 1987; Ohemeng *et al.*, 1993) or interference with energy metabolism by inhibition of NADH-cytochrome *c* reductase, reducing the energy required for active uptake of various metabolites and biosynthesis of macromolecules (Haraguchi *et al.*, 1998; Cushnie and Lamb, 2011). Licochalcone A (Qiu *et al.*, 2010a) or thymol (Qiu *et al.*, 2010b) perform inhibitory effect on α -hemolysin synthesis through inhibition of *agrA* transcription, one of the components of the *agr* global regulatory system of *S. aureus*. Besides influence on *agr* global regulatory system, licochalcone E also directly inhibits expression of *hla*-gene encoding α -hemolysin synthesis (Zhou *et al.*, 2012). Accordingly, it is possible that chalcones also inhibit production of α -hemolysin through mechanisms proposed by Qui *et al.* and Zhou *et al.* Further studies are necessary to elucidate the precise mechanisms of inhibitory effect of tested chalcones on hemolytic activity of α -hemolysin.

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Molecular Methods for Identification of Monophasic *Salmonella* Typhimurium Strains

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Abstract

Two molecular biology methods were used to differentiate *Salmonella enterica* 1,4,[5],12:i:- strains: “*Salmonella* Check&Trace microarray” (CT) and multiplex PCR (mPCR). For 92 strains in CT result “*Salmonella* 1,4,[5],12:i:-” were obtained. Those strains were confirmed in mPCR as monophasic *fljB*-lack *Salmonella* Typhimurium. For 17 strains, which in CT assay were recognized as *Salmonella* Typhimurium, the same identification was obtained in mPCR. Reference *Salmonella* strains: Lagos, Agama, Tsevie, Gloucester and Tumodi in CT were recognized as *Salmonella* genovar, in mPCR – as *Salmonella* O:4, H:i other than *Salmonella* Typhimurium, the same like *Salmonella* Farsta, recognized incorrectly in CT as *Salmonella* Typhimurium.

Key words: microarray, molecular methods, monophasic *Salmonella* Typhimurium, multiplex PCR, serotyping

Salmonella is a significant etiologic agent of bacterial intestinal infections in Poland and other European Union countries. According to the data published by NPHI-NIH Department of Epidemiology in the report “Infectious diseases and poisonings in Poland in 2013”, 7577 cases of human *Salmonella* infections were notified in 2013 in our country. Among the most common *Salmonella* serovars, *Salmonella* Enteritidis and *Salmonella* Typhimurium were isolated. Basing on the data collected by European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), similar situation is also observed in other European countries, but as far as the end of the last century *Salmonella* Enteritidis were responsible for about 80% of cases of salmonellosis, whereas since 2000 systematic decrease in the frequency of isolation of serovar Enteritidis is observed. At the same time, especially in recent years, increase of infections caused by *Salmonella* Typhimurium is notified. This is a quite worrisome situation, because in opposition to *Salmonella* Enteritidis strains, which mostly are antimicrobial-sensitive, among *Salmonella* Typhimurium isolates about 60% of strains isolated from people have higher antimicrobial resistance, especially for ampicillin, tetracycline and sulphonamides. Moreover, at the end of the '90s in a few European countries and USA outbreaks of food

poisoning caused by *Salmonella enterica* subsp. *enterica* 1,4,[5],12:i:- were observed. Using methods of molecular biology, this etiologic agent was finally recognized as *Salmonella* Typhimurium. Since the 90's increasing incidence of monophasic *Salmonella* strains with antigenic formula 1,4,[5],12:i:- has been observed. According to the EFSA/ECDC data, *Salmonella* 1,4,[5],12:i:- has been the third most common *Salmonella* serovar isolated from human samples in 2011. 90 % of these strains were resistant to ampicillin, streptomycin, tetracycline and sulphonamides in (EFSA, 2012).

An increasing number of *Salmonella* O:4 strains with “i” flagellar antigen, non-agglutinating with any antisera for second-phase of H antigen is also observed in Poland. There is no precise information, of which *Salmonella* serovar this monophasic variant is. It could be monophasic *Salmonella* Typhimurium or one of the other serovars, belonging to the same serogroup, which could have such antigenic formula, *i.e.* *Salmonella* Lagos, *Salmonella* Agama, *Salmonella* Farsta, *Salmonella* Tsevie, *Salmonella* Gloucester or *Salmonella* Tumodi (Grimont and Weill, 2007).

Salmonella serovars mentioned above could be recognized in a few ways. The most popular is the conventional serotyping method – determining the presence of somatic and flagellar antigens by slide agglutination

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using specific antisera (Szych and Madajczak, 2010). Then serovar is identified according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007). Unfortunately, this method has many limitations, connected with antisera quality or atypical forms of *Salmonella* antigens. Such deviation like monophasic form or roughness could obstruct the determination of antigenic structure and serovar identification. For that reason many alternative – molecular biology based methods have been developed, however not all of them are routinely used. The most precise, and the same most complicated, are microarray-based and sequencing-based methods (Achtman *et al.*, 2012; Braun *et al.*, 2012; Franklin *et al.*, 2011). Some of those methods found application in commercially available tests, like microarray based “Salmonella Check&Trace” (Check-Points BV, Netherlands) or bead-based suspension array – xMAP® Salmonella Serotyping Assay (Luminex, USA) (Fitzgerald *et al.*, 2007; Jean-Gilles Beaubrun *et al.*, 2014; Wattiau *et al.*, 2008). All these methods could be used for differentiation of *Salmonella* serovars with O:4 and H:i antigens, with undetectable second phase flagellar antigen by traditional methods, but they are a little bit too complicated. For that reason, EFSA in their scientific opinion proposed the simple multiplex-PCR-based method for identification and differentiation of *Salmonella* Typhimurium and its monophasic variant 4,[5],12:i:-. In this method two genetic markers are used: intergenic region of the phase I flagellin gene cluster *fliB-fliA* and variable region of *fljB* gene, which encodes II-phase flagellar antigen (EFSA BIOHAZ, 2010).

In this study two molecular biology methods were used to differentiate *Salmonella* strains belonging to the O:4 serogroup and sharing the same first flagellar antigen H:i: microarray-based method and recommended by EFSA multiplex PCR.

One hundred and ten *Salmonella enterica* subsp. *enterica* strains with O:4 and H:i antigens have been used for this study. Samples were collected in 2007–2012 years in regional Epidemiological Sanitary Stations in Poland and in a private company from the food-quality sector. Moreover, the reference strain *Salmonella* Typhimurium ATCC 700720 has been used as well as *Salmonella* Lagos, *Salmonella* Agama, *Salmonella* Farsta, *Salmonella* Tsevie, *Salmonella* Gloucester and *Salmonella* Tumodi strains obtained from the National *Salmonella* Centre (Gdańsk, Poland). All strains used for study were reidentified according to the routinely used procedure, based on the determination of biochemical features in classical homemade tube tests and serovar identification by slide agglutination using specific antisera (Biomed Kraków, Poland; Immunolab, Poland; Staten Serum Institute, Denmark) according to the White-Kauffmann-LeMinor scheme (Grimont and Weill, 2007; Szych and Madajczak, 2010). Moreover,

the phase inversion was performed to obtain II-phase flagellar antigen in accordance with routine procedure (Szych and Madajczak, 2010).

To perform Microarray-based method, the commercially available test “Salmonella Check&Trace microarray” (CT) (Check-Points BV, Netherlands) was used. Assay was performed according to manufacturer instruction. In multiplex PCR the *fljB* gene fragment and intergenic region of the phase I flagellin gene cluster *fliB-fliA* fragment were detected according to the procedure recommended by EFSA (EFSA BIOHAZ, 2010). For *Salmonella* Typhimurium two fragments were expected: 1389 bp for *fljB* gene and 1000 bp for intergenic region. For monophasic *Salmonella* Typhimurium only one product was expected – 1000 bp for intergenic region. Other O:4 strains sharing the “i” antigen have two fragments 1389 bp (*fljB*) and 250 bp (intergenic region).

One hundred and forty six *Salmonella enterica* subsp. *enterica* O:4 strains, that harbor H:i antigen were submitted to the laboratory where this study was performed. After the reidentification process 110 strains were selected for further research. For all strains the second phase of the flagellar antigen was not detected, even in the phase inversion process. The presence of O:1, O:5, O:12 somatic antigens was varied. All other reference *Salmonella* strains used in the study (serovars: Lagos, Agama, Farsta, Tsevie, Gloucester, Tumodi and Typhimurium ATCC700720) were recognized correctly. Seventeen of all monophasic *Salmonella* strains used in the study were recognized in CT assay as *Salmonella* Typhimurium. For 16 strains in *Salmonella* Typhimurium group, “*Salmonella* Typhimurium (10909)” result was obtained and for one strain (PZH 113/07) “*Salmonella* Typhimurium (11933)”. Ninety two strains were recognized as “*Salmonella* 1,4,[5],12:i:- (2717)”. For one strain (PZH 258/12) the result “*Salmonella*, genovar 10397” was obtained. For the selected strains from the *Salmonella* Typhimurium group mentioned above, a more precise phase inversion process was performed. Highly concentrated, not-commercially available, H:i antiserum have been used. For one such strain, after third round of phase inversion with addition 0.5 ml of antiserum, a positive reaction with II-phase antigens H:1,2 was obtained (strain no PZH 113/07). For three more strains phase inversion was unsuccessful. For reference non-*Salmonella* Typhimurium strains in CT assay “*Salmonella*, genovar” result was obtained with various code. One of them – *Salmonella* Farsta was incorrectly recognized as “*Salmonella* Typhimurium, 10909”. The CT assay for this strain was repeated with the same faulty result. Detailed information about CT assay results are presented in Table I.

Ninety two *S. enterica* subsp. *enterica* 1,4,[5],12:i:- strains, recognized in both (serotyping and microarray)

Table I
Summarized results of CT microarray and multiplex PCR.

Final serovar / antigenic formula identification	No	CT microarray result	Multiplex PCR result
<i>S. enterica</i> 1,4,[5],12:i:-	16	<i>Salmonella</i> Typhimurium (10909)	S. Typhimurium
S. Typhimurium*	1	<i>Salmonella</i> Typhimurium (11933)	
S. Typhimurium ATCC 700720	1	<i>Salmonella</i> Typhimurium (10909)	
<i>S. enterica</i> 1,4,[5],12:i:-	92	<i>Salmonella</i> 1,4,[5],12:i:- (2717)	Monophasic S. Typhimurium
<i>S. enterica</i> 1,4,[5],12:i:- **	1	<i>Salmonella</i> , genovar (10397)	<i>Salmonella</i> O:4, H:i other than S. Typhimurium
S. Tumodi	1	<i>Salmonella</i> , genovar (8232)	
S. Gloucester	1	<i>Salmonella</i> , genovar (14390)	
S. Tsevie	1	<i>Salmonella</i> , genovar (15912)	
S. Farsta	1	<i>Salmonella</i> Typhimurium (10909)***	
S. Agama	1	<i>Salmonella</i> , genovar (10762)	
S. Lagos	1	<i>Salmonella</i> , genovar (9865)	

* Strain PZH 113/07 initially identified as *S. enterica* 1,4,[5],12:i:-; ** Strain PZH 258/12; *** Incorrect identification

methods as *S. enterica* 1,4,[5],12:i:- in multiplex PCR were recognized as monophasic *Salmonella* Typhimurium, with single 1000 bp product for *fliB-fliA* intergenic region. Strain PZH 258/12 was also recognized as monophasic *Salmonella* Typhimurium. Strain PZH 113/07 was found to be *Salmonella* Typhimurium with two specific multiplex PCR products. The same result was obtained for 16 strains with difference in serotyping and CT microarray and for reference strain *Salmonella* Typhimurium ATCC 700720. For all reference non-*Salmonella* Typhimurium strains, two DNA fragments were detected – 1389 bp and 250 bp, as expected.

According to the data published by EFSA/ECDC monophasic *Salmonella* Typhimurium is one of the most frequent serovars isolated from human, animal food and feed samples in the last years (EFSA and ECDC, 2014). Unfortunately it is impossible to distinguish it with conventional methods from other monophasic *Salmonella* from O:4 group, which harbor H:i antigen. It could be done only using molecular biology methods, which allow to identify *Salmonella* serovar, when classical serologic methods fail (Fitzgerald *et al.*, 2007; Franklin *et al.*, 2011; Junia Jean-Gilles Beaubrun *et al.*, 2012; Wattiau *et al.*, 2008). Those methods were not developed only for monophasic *Salmonella* Typhimurium identification, but for broad spectrum of *Salmonella* serovars. They are also not routinely used, mostly because of high hardware requirements and high costs for single test. For that reason other techniques, mostly simple PCR-based, were developed, which allow to support conventional serotyping technique, especially in case of difficult strains (monophasic or rough). For example, Herrera-Leon *et al.* developed multiplex PCR for identification of most common phase-I flagellar antigens (Herrera-

Leon *et al.*, 2004). Moreover, Echeita *et al.* developed a PCR procedure for H1 antigenic complex identification and for other antigens (Echeita *et al.*, 2002; Echeita and Usera, 1998). Both techniques could be used also for monophasic *Salmonella* Typhimurium identification, but under condition – *fliB* gene presence. Present studies, similar to the results of Echeita *et al.* showed that epidemic *S. enterica* subsp. *enterica* 1,4,[5],12:i:- strains are *fliB*-negative variants of *Salmonella* Typhimurium (Echeita *et al.*, 2001). For that reason other techniques must be used for such strains identification, for example EFSA-recommended two-genes multiplex PCR (EFSA BIOHAZ, 2010). Check&Trace microarray mentioned previously is also useful for monophasic *Salmonella* strains serotyping, including *S. enterica* subsp. *enterica* 1,4,[5],12:i:- serovar (Check-Points BV, 2014). Validation of this test was the subject of a few studies, also for diagnostically difficult *Salmonella* strains (Jean-Gilles Beaubrun *et al.*, 2014; Madajczak and Szych, 2010; Wattiau *et al.*, 2008). In previous studies usefulness of CT assay was confirmed, also for monophasic strains, but not for monophasic *Salmonella* Typhimurium (Madajczak and Szych, 2010). Present study, confirmed similar capability of both tests to differentiate *Salmonella* Typhimurium from non-*Salmonella* Typhimurium strains, however multiplex PCR could also confirm the presence of *fliB* gene sequence, which encode second phase of flagellar antigen, what give more information needed for complete strain identification. Together with *fliB-fliA* intergenic region typing it gives information about affinity to group of *Salmonella* serovars, which harbor O:4 and H:i antigens. Moreover CT microarray gave faulty identification for *Salmonella* Farsta, correctly recognized in multiplex PCR. Unfortunately it was impossible to validate both tests for identification of monophasic *Salmonella* strains O:4 and H:i. Such

strains were not isolated during the 6 years of our study, but according to principle of operation of both tests, it is probable, that they could be correctly recognized in multiplex PCR and unrecognized in CT assay.

Comparing these two methods, another important factor should be taken into consideration, that is the costs of equipment needed to perform both tests. CT assay needs dedicated only for this technique (Tube Array) reader. Multiplex PCR could be performed with standard thermocycler available in many laboratories.

Multiplex PCR seems to be a better method than Check&Trace microarray for differentiation of monophasic *Salmonella* Typhimurium and other *Salmonella* which harbor O:4 and H:i antigens.

Acknowledgments

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Probable Interspecies Transfer of the *bla*_{VIM-4} Gene between *Enterobacter cloacae* and *Klebsiella pneumoniae* in a Single Infant Patient

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Abstract

We report the interspecies transfer of the *bla*_{VIM-4} gene in MBL-producing *Enterobacter cloacae* and *Klebsiella pneumoniae* isolates from a newborn patient who had received meropenem therapy. We show evidence that gene *bla*_{VIM-4} was transmitted as a part of the class-1 integron on a ca. ~90 kb conjugative plasmid. High homology of nucleotide sequence was observed between the integron found in VIM-4 producing *E. cloacae* and *K. pneumoniae* strains tested and class-1 integrons previously reported in *Pseudomonas aeruginosa* from Hungary and Poland. This finding may suggest *P. aeruginosa* as a potential source of acquired VIM-4 in *Enterobacteriaceae*.

Key words: *Enterobacteriaceae*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, MBL, VIM-4

Carbapenemase-producing bacteria are an increasing international public health threat (Canton *et al.*, 2012). Metallo- β -lactamases (MBLs), which confer resistance to carbapenems have been detected worldwide in Gram-negative non-fermenting bacteria. More recently, MBLs became frequently reported among *Enterobacteriaceae*, in which these enzymes are encoded by genes located in transferable plasmids (Vatopoulos, 2008). Besides NDM (New Delhi MBL), the VIM-type (Verona integron-encoded MBLs) enzymes are the most important MBLs for epidemiological dissemination and clinical relevance. After the first detection of plasmid acquired VIM-4 in *Pseudomonas aeruginosa* in Greece in 2001 (Pournaras *et al.*, 2002), VIM enzymes have been soon reported in many countries, including Poland, where VIM-2 and VIM-4 producing *Pseudomonas* spp. were observed (Fielt *et al.*, 2006; Patzer *et al.*, 2009). To the best of our knowledge, the first case of VIM-1 group producing *Klebsiella pneumoniae* in Poland was reported in 2010 by Sękowska from a 61 year-old patient admitted at a Teaching Hospital in Bydgoszcz, Poland (Sękowska *et al.*, 2010a,

2010b). Until now, VIM-1, VIM-4 and VIM-35-producing *Enterobacteriaceae* were detected in our country (Castenheira *et al.*, 2014).

Herein, we report VIM-4-producing *Enterobacter cloacae* and *K. pneumoniae* strains isolated in 2010 from newborn patient. We also show evidence arguing for the interspecies transmission of a conjugative plasmid carrying the *bla*_{VIM-4} gene located as a part of class 1 integron.

A one month-old male-newborn, that had been hospitalised in an tertiary maternity hospital was admitted to Intensive Care Unit (ICU) of a secondary children hospital in Warsaw, Poland on March 18, 2010 due to prematurity with extremely low birth weight and necrotising enterocolitis. During his stay in the ICU (till July 15, 2010), the newborn-patient received a broad-range antimicrobial-regimen, including meropenem. On 16 and 21 July, 2010, when the patient was at a surgery ward, *E. cloacae* and *K. pneumoniae* isolates with reduced susceptibility to ertapenem were collected. The both isolates were found to produce MBL-type carbapenemase (Table I) as shown by use of the phenotypic

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Table I
Characteristics of VIM-4 producing clinical isolates of *E. cloacae* and *K. pneumoniae* together with the respective *E. coli* electrotransformants.

Strain	Species	Specimen	MBL ^b	MIC ^a (mg l ⁻¹) of:											
				CTX	CAZ	FEP	ATM	ETP	IPM	MEM	GEN	AMK	CIP	CST	TGC
ECV	<i>E. cloacae</i> (16.07.2010)	wound	+	> 256	48	32	64	0.75	0.5	0.19	64	16	1.5	0.5	4
KPV	<i>K. pneumoniae</i> (21.07.2010)	stool	+	24	6	1.5	0.047	0.75	0.75	0.38	1	16	0.125	0.38	3
ET	<i>E. coli</i> DH5 α	NA	-	0.023	0.25	0.016	0.032	0.004	0.125	0.016	0.125	0.25	0.023	0.094	0.38
ET-pEc90	<i>E. coli</i> DH5 α	NA	+	12	6	0.38	0.047	0.125	0.75	0.38	0.5	12	0.012	0.125	0.38
ET-pKp90	<i>E. coli</i> DH5 α	NA	+	16	4	0.38	0.032	0.19	0.75	0.125	0.25	6	0.016	0.125	0.38

^a Abbreviations: AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; FEP, cefepime; ETP, ertapenem; GEN, gentamicin; IPM, imipenem; MEM, meropenem; TGC – tigecycline. NA – not applicable.

^b MBLs were detected as described previously (Franklin *et al.*, 2006; Tato *et al.*, 2010).

Table II
Primers used for PCR mapping and sequencing of *bla*_{VIM} genes and their environment

Primer	Sequence 5'-3'	Reference
VIM-F	ATCATGGCTATTGCGAGTCC	In this study
VIM-R	ACGACTGAGCGATTTGTGTG	
5'-CS	GGCATCCAAGCACAAGC	Zhao <i>et al.</i> (2001)
3'-CS	AAGCAGACTTGACTGAT	
VIMA	CAACTCATCACCATCACGGACAAT	In this study
VIMB	GGGGAGCCGAGTGGTGAGTATC	In this study

tests described previously by Franklin *et al.* (2006) and Tato *et al.* (2010). Detection of the *bla*_{VIM} gene and characterization of its environment were performed using conventional PCR mapping and DNA-sequencing with the primers listed in Table II.

The *bla*_{VIM} gene was located on *ca.* ~90kb plasmids named: pEc90 and pKp90, as shown by DNA/DNA Southern-blot with VIM-probe. The Southern-blot was conducted as previously described (Wardak *et al.*, 2007). Both plasmids were separately transferred to *Escherichia coli* DH5 α by electro-transformation (Zacharczuk *et al.*, 2011), and MICs of the both transformants were determined (Table II). Restriction endonuclease *Pst*I profiles of the pEc90 and pKp90 from the transformants were indistinguishable. DNA sequencing of the *bla*_{VIM} genes from pEc90 and pKp90 revealed VIM-4 variant. The ability for conjugational transfer of the both plasmids was exhibited, using a conventional liquid-medium mating-test with rifampicin-resistant *E. coli* CSH26 recipient strain. PCR mapping and DNA-sequencing (Zhao *et al.*, 2001) shown the *bla*_{VIM-4} gene was located as a part of the class 1 integron in pEc90 and pKp90. The class-1 integron found in the both plasmids carried two resistance gene-cassettes, where in the first position was the *aac*(6')-Ib gene (also named *aacA4*) followed by *bla*_{VIM-4}. Subsequent DNA-sequence analysis

using blast-n (NCBI) revealed that the class-1 integron reported herein has 100% homology to integron found in *P. aeruginosa* (access. no. GU181265) from Hungary. Moreover, the class-1 integron reported herein in carbapenemase non-susceptible isolates of *E. cloacae* and *K. pneumoniae*, was probably closely related to the integron from *P. aeruginosa* (access. no. AJ585042) from Poland. The only difference were two point-mutations (T1670G and C1671T).

In conclusion, the evidence collected in our laboratory may strongly argue for the lateral transfer of the *bla*_{VIM-4} gene between *E. cloacae* and *K. pneumoniae* isolates from the same patient. Similar findings were reported in 2002, by Luzzaro and colleagues (Luzzaro *et al.*, 2004), who recovered VIM-4 producing strains of *E. cloacae* and *K. pneumoniae* from a 72-year-old patient that had received 4-weeks of imipenem therapy. Moreover, the nucleotide sequence of the class 1 integron that carries the *bla*_{VIM-4} gene in the both *Enterobacteriaceae* strains described in this paper may suggest *P. aeruginosa* as a potential source of VIM-4.

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Five Years' Evaluation of the BD ProbeTec System for the Direct Molecular Detection of *Mycobacterium tuberculosis* Complex in Respiratory and Nonrespiratory Clinical Samples

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Abstract

In this study, *Mycobacterium tuberculosis* complex was detected by BD ProbeTec ET system in 4716 respiratory and 167 nonrespiratory samples [mostly (98%) smear negative]. Sensitivity, specificity, positive and negative predictive values were 81.8%, 98.3, 85.1 and 97.9 for respiratory and 100%, 96.2, 64.7 and 100, for nonrespiratory samples, respectively. Among 149 (3.1%) ProbeTec DTB positive and culture negative samples, 72 (65 respiratory and seven nonrespiratory) (48.3%) were recovered from the patients who were evaluated as having TB infection. The system has been found as useful in early diagnosis of tuberculosis infection in association with the clinical, radiological and histopathological findings.

Key words: BD ProbeTec ET, extrapulmonary, molecular detection, pulmonary, tuberculosis

According to the World Health Organization (WHO), tuberculosis (TB) has been still an important problem all over world (WHO, 2013a). The definitive TB diagnosis depends on the results of microbiological tests in addition to the clinical, radiological and histopathological data and the mycobacterial culture has been accepted as the gold standard. As the WHO started DOTS [Directly Observed Therapy Strategy (plus)] (WHO, 1999; Gupta *et al.*, 2003) for treatment of TB disease, early and accurate diagnosis has become an important issue to take precautions and to initiate treatment plan in the hospital clinics for chest diseases. Although microscopic examination and culture are major microbiological tests for TB diagnosis, the low sensitivity of microscopy and the necessity of long incubation time for culture are the most significant limitations. Therefore, nucleic acid amplification (NAA) techniques have been used for early and differentiative detection of causative mycobacteria in clinical samples and also to support the clinical and radiological diagnosis in patients with presumptive *Mycobacterium tuberculosis* infection (CDC, 2009). One of the automated systems widely used is the BD ProbeTec ET which is

based on the strand displacement amplification (SDA) technology. The system utilizes homogenous SDA technology as the amplification method and fluorescent energy transfer (ET) as the method of detecting the presence of *Mycobacterium tuberculosis* complex directly from clinical samples.

The aim of this study was to evaluate the performance of BD ProbeTec ET *M. tuberculosis* complex (DTB) Assay according to the laboratory data of five years in accordance with the clinical diagnosis. The clinical evaluation of patients for TB diagnosis and follow-up procedures were made by specialist physicians according to the WHO and national guidelines (WHO, 2013b).

A total of 4883 samples [4716 respiratory (4626 bronchial aspiration and 90 sputum), and 167 nonrespiratory (NR) (102 fine needle aspiration biopsy (FNAB), 27 urine, 15 cerebrospinal fluid (CSF), 11 pleural fluid, 2 gastric lavage, 2 surgical material and 8 various sterile body fluids or pus)] recovered from 4304 patients between October 2009 and October 2013 were analyzed in the Microbiology Laboratory of Izmir Training and Research Hospital for Chest Diseases and Chest

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Surgery which has been a regional reference hospital for TB patients at the Aegean Coast of Turkey (West Anatolian Region). The patients included in the study were evaluated as having suspicious TB infection. An acid-fast smear preparation, mycobacterial cultivation, identification and molecular detection were applied to each sample.

Mycobacterial cultivation was performed by MGIT 960 system (BD Biosciences, Sparks, MD, USA) and in Lowenstein-Jensen slants. Mycobacterial identification was performed by conventional methods (Koneman *et al.*, 1992), BACTEC 460 p-nitro- α -acetyl amino- β -hydroxypropionophenone (NAP) or BD immunochromatographic test. Additionally, commercially available PCR based reverse hybridization (Line Probe Assay = LiPA) kits were used for further identification of nontuberculous mycobacteria in species level.

Rapid molecular detection and identification for each sample was performed by the BD ProbeTec ET *M. tuberculosis* complex (DTB) Assay on the basis of BD ProbeTec ET system according to the manufacturer's recommendations. The test system has utilized homogenous SDA technology as the amplification method and fluorescent ET as the method of detecting the presence of *M. tuberculosis* complex DNA directly from clinical specimens. All calculations were performed automatically by the instrument software. Results were reported through an algorithm as positive, negative, or indeterminate. The molecular assays with the discrepant results according to the culture and which were considered as false positive or had cross-contamination were repeated using frozen aliquots of the samples. The same result which was taken twice (either negative or positive) repeatedly was accepted as the final result in these discrepant assays. Specimens with invalid results showing inhibition of internal control (IC) and sample amplification were also retested with the dilution of 1/100. Totally, IC and sample amplification was not observed in 6 (0.12%) samples which were AFB negative and evaluated as inhibition. These samples gave valid results with the dilution of 1/100.

Among 4883 specimens tested, 4784 (98%) and 99 (2%) were smear negative and positive with acid-fast staining, respectively. ProbeTec DTB tests of 193 (4%) samples were repeated because of discrepancy. After evaluation of test results, 149 (136 respiratory, 13 NR) (3.1%) samples were culture negative and ProbeTec DTB positive, whereas 90 respiratory (1.8%) samples were culture positive and ProbeTec DTB negative. ProbeTec DTB negative and culture positive samples were also confirmed as TB by clinical evaluation. Among ProbeTec DTB positive and culture negative samples (n = 149), 72 (65 respiratory, 6 FNAB and 1 pleural fluid) (48.3%) samples were recovered from the patients who were evaluated as having TB infection

and applied anti-TB treatment according to the clinical data and/or positive mycobacterial cultures taken from the other separate samples. Sixty eight of patients had TB diagnosis with clinical evaluation solely, while four patients had additional positive mycobacterial cultures taken from other separate samples. The rest of the samples (70 bronchial aspiration and 1 sputum; respiratory and 2 urine, 2 FNAB, 1 abscess and 1 gastric lavage; NR) were obtained from the patients with a diagnosis other than active TB infection [*i.e.* 69 respiratory samples obtained from patients with pneumonia (n = 20), lung malignancy (n = 20), COPD (n = 8), past TB infection (n = 6), sarcoidosis (n = 3), hemoptysis (n = 4), hydatidosis (n = 2), empyema (n = 1), asthma (n = 1), bronchiectasis (n = 1), Churg-Strauss syndrome (n = 1), interstitial lung disease (n = 1), silicosis (n = 1) and 6 NR samples obtained from patients with urinary tract infection (n = 2), lung malignancy (n = 1), sarcoidosis (n = 1), skin abscess (n = 1) and pneumonia (n = 1)]. Additionally, two bronchial aspiration which were taken from patients with diagnosis of extrapulmonary TB (pleurisy and lymphadenitis) were ProbeTec DTB positive and culture negative as well. In 13 respiratory samples, nontuberculous mycobacterial growth was positive in culture. Among these, two samples (sputum and bronchial aspiration) were found as false positive and the other 11 samples were found as negative by ProbeTec DTB test. Distribution of culture and ProbeTec DTB test results in smear negative and positive samples according to the sample type has been shown in Table I. Sensitivity, specificity, positive and negative predictive values for ProbeTec DTB test in clinical samples when compared with the culture and with the culture in combination with the clinical diagnosis have been shown in Table II.

ProbeTec ET is one of the widely used semi-automated NAA systems based on SDA technology. The semi-automated systems like ProbeTec ET, Cobas Taqman MTB (Roche, Germany), Gen-Probe Amplified MTB Direct Test (Gene-Probe, USA) and recently, some other technologies such as RT-PCR based assays have the advantage of processing larger batch of samples at once. In low-prevalence settings, use of these systems may be more preferable to make a differential diagnosis for TB from many other clinical pictures. Patient population selected for NAA testing in TB can be variable according to the clinical findings, stage of the disease (*i.e.* anti-TB treatment), the incidence of mycobacteria in that region and the experience of the laboratory. Each TB control or treatment program should evaluate the overall costs and benefits of NAA testing in deciding the value and optimal use of the test in their setting. As the incidence of *M. tuberculosis* complex has been reported to be low (app. 20%) in our region (WHO, 2013a), it has been considered that

Table I
Distribution of culture* and ProbeTec DTB test results in smear negative and positive samples according to sample type

Sample type		Smear negative (n = 4784) Culture / BD ProbeTec				Smear positive (n = 99) Culture / BD ProbeTec			
		+/-†	-/+‡	+/+	-/-	+/-†	-/+‡	+/+	-/-
Respiratory (n = 4716)	BA / BAL (n = 4626)	81	130	237	4085	3	1	86	3
	Sputum (n = 90)	6	3	14	61	0	2	3	1
NR (n = 167)	Biopsy [§] (n = 104)	0	8	0	96	0	0	0	0
	Urine (n = 27)	0	2	1	24	0	0	0	0
	CSF (n = 15)	0	0	2	13	0	0	0	0
	PF (n = 11)	0	1	0	10	0	0	0	0
	SBF (n = 8)	0	1	1	6	0	0	0	0
	GL (n = 2)	0	1	0	1	0	0	0	0
Total (n = 4883)		87	146	255	4296	3	3	89	4

Abbreviations: NR: Nonrespiratory, BA: Bronchial aspiration, BAL: bronchoalveolar lavage, CSF: cerebrospinal fluid, PF: Pleural fluid, SBF: Sterile body fluids, GL: Gastric lavage, -: negative, +: positive

* Culture was done by BACTEC 960 system and in Lowenstein Jensen medium.

† BD ProbeTec ET DTB negative and culture positive samples were also confirmed as TB by clinical evaluation.

‡ Among BD ProbeTec ET DTB positive and culture negative samples, 72 (48.3%) samples (65 respiratory, 7 nonrespiratory) were recovered from the patients who were evaluated as having TB infection and applied anti-TB treatment according to the clinical data and/or positive mycobacterial cultures taken from the other separate samples.

§ Biopsy (*i.e.* 102 fine needle aspiration biopsy (FNAB) and two surgical materials).

|| Sterile body fluids (*e.g.* pericardial fluid, wound, ascites)

Table II
Sensitivity, specificity, positive and negative predictive values for ProbeTec DTB test in clinical samples*

Sample Type		Sensitivity (%)		Specificity (%)		PPD (%)		NPD (%)	
		A	B	A	B	A	B	A	B
Smear negative	Respiratory	74.3	78.4	96.9	98.3	65.4	82.3	97.9	97.9
	Nonrespiratory	100.0	100.0	92	96.2	23.5	64.7	100.0	100.0
	Total	74.6	78.8	96.7	98.5	63.6	80.8	98	98
Smear positive	Respiratory	96.7	96.8	N/A	N/A	96.7	100.0	N/A	N/A
All Samples	Respiratory	79.1	81.8	96.8	98.3	71.4	85.1	97.9	97.9
	Nonrespiratory	100.0	100.0	92	96.2	23.5	64.7	100.0	100.0
	Total	79.3	82.2	96.7	98.2	69.8	84.4	97.9	97.9

* Column A indicates the sensitivity, specificity, positive and negative predictive values compared with the culture, whereas column B indicates the values compared with the culture in combination with the clinical diagnosis.

a NAA testing in addition to the conventional culture and automated cultivation systems which is available for batch processing and random access under the same platform would be an ideal solution for rapid and accurate diagnosis and treatment planning.

In previous studies, the sensitivity, the specificity, positive and negative predictive levels of the BD ProbeTec ET were reported within the ranges of 56.7–100, 95.3–100, 59.6–100 and 96–100%, for respiratory, 50–100 and 93.3–98.7, 33.3–80 and 97.8–100%, for nonrespiratory samples, respectively (Abdel-Aziz *et al.*, 2011; Antonenka *et al.*, 2013; Barber, 2008; Barrett *et al.*, 2002; Bergmann and Woods, 1998; Bergmann *et al.*, 2000; Johansen *et al.*, 2002; Karadag *et al.*, 2013; Maugein *et al.*, 2002; Mazzarelli *et al.*, 2003; Pfyffer *et al.*, 1999; Piersimoni *et al.*, 2002; Rüscher-Gerdes and

Richter, 2004; Tu *et al.*, 2011; Wang *et al.*, 2004; Wang *et al.*, 2006). In the present study, the corresponding performance values were found as 81.8, 98.3, 85.1 and 97.9%, for respiratory, and 100, 96.2, 64.7 and 100%, for nonrespiratory samples, respectively. The evaluation of discrepant results in accordance with the other positive cultures and clinical outcome made a low level of increase in the sensitivity and specificity values, but a high level of increase was observed in positive predictive values in respiratory and nonrespiratory samples (Table II). Sensitivity was evaluated as low as with the other studies using different NAA methods in smear negative and nonrespiratory samples whilst the specificity was high. However, this study differs from other studies using BD ProbeTec ET system which reported low sensitivity (25–60%) (Barber, 2008; Barrett *et al.*,

2002; Johansen *et al.*, 2002; Wang *et al.*, 2004; Wang *et al.*, 2006) and positive predictive values (40.9% and 44.7%) (Pfyffer *et al.*, 1999; Wang *et al.*, 2004) for smear negative respiratory samples and higher values of positive predictive values (70–80%) (Abdel-Aziz *et al.*, 2011; Karadag *et al.*, 2013; Rüsç-Gerdes and Richter, 2004) for nonrespiratory samples. The performance values of this system might have been misevaluated due to the low sample population which has been included in these studies. As this study was undertaken with high number of sample population for a long period of time, it was thought that a more precise evaluation was achieved for the diagnostic performance of BD ProbeTec ET in smear negative respiratory samples. In nonrespiratory samples, less number of samples (n = 167) were submitted to our laboratory and a low positivity (n = 17) was found as a natural feature of these samples due to the difficulty in laboratory as well as clinical diagnosis. Thus, in two patients who was evaluated as TB clinically, FNAB samples were negative by both culture and BD ProbeTec. Among thirteen BD ProbeTec positive and culture negative nonrespiratory samples, six fine needle aspiration biopsy and a pleural fluid were recovered from the patients who were evaluated as tuberculosis clinically.

In conclusion, in terms of performance values, the ProbeTec ET system has been found as useful in early diagnosis of pulmonary and extrapulmonary TB infection in association with the clinical, radiological and histopathological findings. Nevertheless, the low positive predictive values also address the need for advanced technologies in addition to the conventional methodologies to provide more precise diagnosis in extrapulmonary TB cases.

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Tuberculosis in Antelopes in a Zoo in Poland – Problem of Public Health

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Abstract

Bovine tuberculosis is an infectious disease that occurs in many species of both domestic and wild animals, as well as those held in captivity. The etiological factor is the acid resistant bacillus (*Mycobacterium bovis* or *Mycobacterium caprae*), which is characterized by the major pathogenicity among mycobacteria belonging to the *Mycobacterium tuberculosis* complex. The material from 8 antelopes from the zoo, suspected for tuberculosis were examined, and *M. bovis* strains were isolated from 6 of them. The spoligotyping method showing spoligo pattern 67676377777600. In Poland, this spoligotype has not been observed so far.

Key words: *Mycobacterium bovis*, antelopes, MTBC, public health, zoo

Mycobacterium bovis is the main cause of tuberculosis in cattle (BTB) and other bovids. It is one of the species belonging to the *Mycobacterium tuberculosis* complex (MTBC) (Rodríguez-Campos *et al.*, 2014). The first complete genome sequence of strain *M. bovis* was described in 2003 (Garnier *et al.*, 2003). *M. bovis* is characterized by natural resistance to pyrazinamide (PZA), one of the most important antibiotics used in the treatment of tuberculosis. PZA resistance phenotype may vary depending on the environment, therefore the test cannot be the basis for species identification (Krajewska *et al.*, 2013). BTB is a quite common zoonotic disease occurring in wild animals kept in zoos and wild animal parks. Tuberculosis caused by members of MTBC has been confirmed, *inter alia*, in Asian elephants (*Elephas maximus*), baboons (*Papio hamadryas*), leopards (*Panthera uncia* and *Panthera pardus*), buffalos (*Bison bonasus*), bison (*Bison bison*), red deers (*Cervus elaphus*) and a sea-lions (*Otaria byrona*) (Thorel *et al.*, 1998; Lewerin *et al.*, 2005; Schmidbauer *et al.*, 2007). This disease is characterized mainly by the symptoms of general weakness, progressive emaciation and respiratory dysfunction. The aim of the study was

to describe the case of tuberculosis in antelopes family (*Addax nasomaculatus*).

The BTB outbreak was detected in the zoo in Chorzów, in 2010. The described herd consisted of 3 males and 5 females, held in the same pavilion. The age of the animals ranged from several months to 10 years (Table I). Incidences of BTB had been reported in that zoo prior to the above mentioned outbreak. Problems with tuberculosis returned in 2010, since two male giraffes were transferred to another zoo, to avoid infection. In October 2010, field veterinarians detected clinical signs of the disease (respiratory track infection and distress) and sudden deaths of the two youngest addaxes (Table I). BTB lesions were observed in the necropsy of both of these antelopes. Lesions were changed as a visible foci of conglomerate lobar pneumonia. The tubercles (from 2 to 8 mm) were observed in the mediastinal lymph nodes with yellowish content in the cut surface. After that incident, the rest of the antelopes were tested using the official bovine PPD, according to the Polish official instruction. All 6 animals were BTB positive, showing a skin fold thickness increase of 4 or more mm and they were euthanized (Table I). The material

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Tabela I

The antelopes family (*Addax nasomaculatus*) and their kinship. Konrad, Mimi and Nora, three antelopes had died earlier – not examined.

Animal name	Data of birth/Sex	Kinship	Place of birth	Clinical symptoms	Tuberculin skin test results
1. TUSIA	2010-03-02 female	Father: Czesio Mother: Matyllda	ZOO Chorzów	respiratory problems	sudden death not tested
2. ARIEL	2010-03-13 male	F: Czesio M: Młoda	ZOO Chorzów	respiratory problems, convulsions	sudden death not tested
3. IWANKA	2000-08-11 female	unknown	ZOO Rotterdam	Zero symptoms	Positive result (4 mm)
4. CZESIO	2004-11-22 male	unknown	ZOO Prague	Zero symptoms	Positive result (4 mm)
5. KOKOLINO	2010-03-15 male	F: Czesio M: Ivanka	ZOO Chorzów	Zero symptoms	Positive result (4 mm)
6. SONIA	2000-12-30 female	F: unknown M: Ivanka (came pregnant)	ZOO Chorzów	Zero symptoms	Positive result (6 mm)
7. MATYLDA	2000-06-08 female	F: Konrad M: Mimi	ZOO Chorzów	Zero symptoms	Positive result (6 mm)
8. MŁODA	2004-05-26 female	F: Konrad M: Mimi	ZOO Chorzów	Zero symptoms	Positive result (8 mm)

for the microbiological examination was collected *post mortem* and appropriate set of lymph nodes and parenchymal organs were derived from a total number of 8. The samples were subjected to laboratory testing, using the standard procedures (Augustynowicz-Kopeć *et al.*, 2011). The GenoType Mycobacterium CM assay (Hain Lifescience, Germany) was performed for the identification of the isolated strains, as recommended by the manufacturer.

Among all the examined antelopes, tuberculous lesions were observed in the thoracic lymph nodes in 5 animals. After 6 weeks a visible growth of acid-fast bacilli in the slants with tissue material from 6 animals was found. The final identification and genotyping of the strains was based on spoligotyping analysis (Kamerbeek *et al.*, 1997). The spoligotyping method indicated that all the isolated strains belong to *M. bovis*, showing a spoligo pattern 676763777777600, which is described in an international spoligo database SpolDB4 as BOV 820. Tuberculosis is a highly contagious disease, transmitted mainly by air passages. The results confirmed the spreading of tuberculosis in a herd of antelopes.

The first case of tuberculosis in a Polish zoological garden was described in 1994 by Kalicki and Rutkowiak. Over the past 20 years, BTB infection was diagnosed in 39 animals belonging to 9 species, including: Alpaca (*Vicugna pacos*), Kob Deffasa (*Kobus ellipsiprymnus deffasa*), Elands (*Taurotragus oryx*), European bison (*Bison bonasus*), Gnu (*Connochaetes taurinus*), Reticulated giraffe (*Giraffa camelopardalis reticulata*), Sitatunga (*Tragelaphus spekei*), Tapir Anta (*Tapirus terrestris*) and Vietnamese pot-bellied pig (*Sus bucculentus*) (Pavlik *et al.*, 2002; Kalicki and Krajewska, 2011; Augustynowicz-Kopeć *et al.*, 2011). The international database SpolDB4 contains 47 strains of the spoligotype

identical with those isolated in antelopes. Among the European strains, 29 originated from Denmark, 6 from Germany, 3 from France and only one from Norway. The SpolDB4 provides information only about the number of the registered strains, reported from different countries. The database stores no information about the clinical material or whether it came from animals or humans.

In the SITVIT WEB, only the strains of 676763 777777600 spoligotype, isolated in Germany, are additionally described as originated from cattle.

Only in the case of the 3 strains isolated in France their animal origin is given, however there is no information on what specific animal species they refer to (Haddad *et al.*, 2001), and even the original data presented in the paper does not make it possible to identify the animal species. For the other strains described in the published literature, there is no information indicating their source of isolation. In Poland, this spoligotype has not been observed so far. It seems that with exchange, the problem of tuberculosis returned to the Polish zoos. As regards the case of the confirmation of the infection with *M. bovis* at the zoo, the thorough disinfection of the cages were kept. All the staff should be examined as a person "of contact" by a pulmonologist. The workers of the zoo should have a tuberculin skin test, a chest X-ray and they should be interviewed carefully as regards the occurrence of subfebrile temperature, weaknesses, cough, *etc.* Also, it should be checked if any of the staff suffered from tuberculosis in the past. Perhaps, the sick human is the source of infection. In Poland, the number of tuberculosis patients is slightly lower than in the previous year. The incidence of tuberculosis in Poland is still higher than the average in the European Union countries, Norway and Iceland

(Korzeniewska-Koseła, 2014). The occurrence of the new multi-drug resistant strains complicates the already long-lasting treatment, which sometimes even leads to the death of the patient (Kruczak and Niżankowska-Mogilnicka, 2009; Kozińska *et al.*, 2011). Bovine tuberculosis in zoos, should be reported to the sanitary and epidemiological stations for the sake of public health protection. Early diagnosis in both humans and animals constitutes a very important element of success in the treatment of such dangerous a disease.

Human tuberculosis caused by *M. tuberculosis* and bovine bacillus (*M. bovis* and *M. caprae*) provide a very similar clinical picture, therefore, a well-organized and equipped microbiology laboratory is responsible for the identification of microorganisms (Augustynowicz-Kopeć and Zwolska, 2010). The main transmission route of the human infection with *M. bovis* is usually raw milk from infected cows or a direct contact with an infected animal, with people visiting zoological gardens being a good example of the latter (van der Heever, 1984; Okolo, 1992; Stone *et al.*, 2011).

The data concerning human tuberculosis caused by bovine bacillus (*M. bovis* and *M. caprae*) comes from 25 EU countries, except Greece and France (Osek and Wiczorek, 2013). In 132 confirmed cases of human tuberculosis in the European Union, the greatest numbers were observed in Germany (38 people), the UK (31 cases) and Spain (22). Thirteen EU countries did not report any bovine tuberculosis in humans. As regards the countries outside the EU, 13 cases were noted in Switzerland and 2 cases in Norway. In Poland, bovine tuberculosis in humans has not been diagnosed. The World Health Organization (WHO) estimates that in the countries where human tuberculosis is poorly supervised, 1% of the disease in humans is caused by bovine bacillus.

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Drug Resistance and Population Structure of *Mycobacterium tuberculosis* Beijing Strains Isolated in Poland

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Abstract

In total, 1095 *Mycobacterium tuberculosis* clinical isolates from 282 patients with drug-resistant and 813 with drug-sensitive tuberculosis (TB) in Poland during 2007–2011 were analysed. Seventy-one (6.5%) patients were found to have strains of Beijing genotype as defined by spoligotyping. The majority of patients were Polish-born; among foreign-born a large proportion came from Chechnya and Vietnam. Analysis showed strong associations between Beijing genotype infection and MDR, pre-XDR and XDR resistance, with a considerable relative risk among new patients, suggesting that this is due to increased spread of drug-resistant strains rather than acquisition of resistance during treatment.

Key words: Beijing family, drug resistance, genotype, spoligotyping

This is the first study on the genetic diversity, drug resistance and population structure of Beijing-TB in Poland. The study included a total of 1095 (~4% all bacteriologically confirmed cases) *Mycobacterium tuberculosis* isolates collected in 2007–2011 in the National TB Reference Laboratory (NTRL) at the National Tuberculosis and Lung Diseases Research Institute in Warsaw. Primary isolation, species identification, and drug susceptibility testing (DST) were done at regional mycobacteriology laboratories. The isolates were subcultured and sent to NTRL, where confirmatory identification and DST were performed. Available demographic data was collected for all patients, such as age, gender, and country of origin. Genomic DNA was obtained from *M. tuberculosis* colonies on L-J slants by the cetyltrimethyl-ammonium bromide (CTAB) method (van Embden *et al.*, 1993).

All isolates were characterized by spoligotyping with a commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands) according to the instructions provided by the manufacturer and as described previously (Kamerbeek *et al.*, 1997).

The patterns obtained by spoligotyping were compared by visual examination and by sorting the results in BioNumerics software version 5.10 (Applied Maths, Kortrijk, Belgium). The spoligotypes were also compared with those contained in the international database SpolDB4 (Brudey *et al.*, 2006). The isolates of the

Beijing genotype were defined by showing hybridization to eight or nine (absent the 37 spacer) spacers between spacers 35 and 43, and showing the absence of hybridization to spacers 1–34 (Kremer *et al.*, 2004; Mokrousov *et al.*, 2002).

Of the 71 (6.5%) strains that were defined by spoligotyping to be of the Beijing genotype, 61 (86%) had all the characteristic spacers from 35 to 43, corresponding to the shared type ST1, and 10 (14%) isolates lacked spacer 37, corresponding to ST265.

Of the 1024 patients with non-Beijing strains, 1018 (99.4%) were born in Poland and 6 were (0.6%) born abroad. Two-hundred twenty-four (21.9%) were infected with drug-resistant (DR) (222 Polish-born, 2 foreigners) and 800 (78.1%) with drug-sensitive (DS) strains (796 Polish-born, 4 foreigners). Of the 71 patients with Beijing strains, 42 (59.2%) were born in Poland. Five of them (11.9%) were infected with DS and 37 (88.1%) were infected with DR strains. Among 29 (40.8%) Beijing strains isolated from foreigners, 8 (27.6%) were DS and 21 (72.4%) were DR strains. Among Polish-born, the majority (57.1%) of patients with Beijing strains were in the 45–64 age group; among foreigners the majority were in the 15–34 age group (58.6%) (Table I).

Of the 29 patients with Beijing strains who were foreign-born, a large proportion (93%) came from Europe and Asia; the majority of these patients were

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Table I
Key data on patients with Beijing – TB

	Polish-born	Foreign-born % (no. of patients)	Total
Age group			
0–14	2.4 (1)	10.3 (3)	5.6 (4)
	2.4 (1)	24.1 (7)	11.3 (8)
	7.1 (3)	34.5 (10)	18.3 (13)
15–24	14.3 (6)	13.8 (4)	14.1 (10)
	33.3 (14)	10.3 (3)	23.9 (17)
	23.8 (10)	3.5 (1)	15.5 (11)
25–34	16.7 (7)	3.5 (1)	11.3 (8)
35–44			
45–54			
55–64			
65+			
Sex			
Male	88.1 (37)	79.3 (23)	84.5 (60)
Female	11.9 (5)	20.7 (6)	15.5 (11)
TB treatment history*			
New patient	57.1 (24)	51.7 (15)	55.0 (39)
Previously treated	31.0 (13)	10.3 (3)	22.5 (16)

* Data missing for 11 foreign-born and 5 Polish-born patients.

from Chechnya (38%) and Vietnam (24%), where the Beijing genotype has been reported to be most prevalent (Merker *et al.*, 2015). The others were from Georgia, Russia, Tibet, India, Ukraine, Slovakia, one from Africa (Nigeria) and one from North America (USA).

Altogether 282 Drug Resistant (DR) and 813 Drug Sensitive (DS) isolates from 1095 patients were identified. The results of drug resistance testing are shown in Table II. While 27 (38%) of the Beijing strains were MDR, 15 (21.1%) pre-XDR and 4 (5.4%) XDR, only 59 (5.8%) of the 1024 non-Beijing strains were MDR, 16 (1.6%) pre-XDR and 14 (1.4%) were XDR during the same period.

Several studies have suggested an association between young age and the Beijing genotype family (Buu *et al.*, 2009; Zanini *et al.*, 2014). Although the Beijing genotype is more common among young patients, we did not find such a correlation in the group of Polish-born patients (9.5%), but observed it among foreign-born males (58.6%) ($P < 0.001$). This suggests that the primary reservoir of Beijing family strains in Poland is young immigrants who came to Poland from the East. It is possible that some of the Beijing strain infections were in fact acquired among Polish-born patients during transmission from young and middle-aged immigrants crossing the Polish border and seeking employment.

An additional argument for the transmission of tuberculosis is the observation that the majority of patients with Beijing-TB belonged to the group of newly diagnosed patients, both among immigrants and Polish-born population.

Numerous molecular epidemiological studies, carried out in various geographical settings, have suggested an association between drug resistance, specifically MDR-TB, and the Beijing genotype strain (Filliol *et al.*, 2002; Tounousova *et al.*, 2004; Drobniowski *et al.*, 2005; Kubica *et al.*, 2005; Park *et al.*, 2005; Caws *et al.*, 2006; Hasan *et al.*, 2006; Victor *et al.*, 2007; Dymova *et al.*, 2014; Maeda *et al.*, 2014). Review of molecular epidemiological data from XDR-TB strains has shown an association between XDR-TB and the Beijing genotype in isolates from South Africa, Estonia, China, Japan and Russia (Iwamoto, 2009; Dheda *et al.*, 2010; Casali *et al.*, 2012).

In this study the prevalence of drug-resistance was significantly higher ($P < 0.001$) in Beijing strains than in non-Beijing isolates (81.7% vs 21.9%). We found strong associations between Beijing genotype infection and MDR, pre-XDR and XDR resistance, with a considerable relative risk among new patients ($P = 0.02$), suggesting that it is increased spread of MDR-TB strains

Table II
Proportion of patients with drug-resistant strains

	Total	% drug resistant (no. of patients with drug resistance)			
		Any drug	MDR	Pre-XDR	XDR
Polish-born					
Beijing	42	88.1 (37)	50 (21)	28.6 (12)	7.1 (3)
Non-Beijing	1018	21.8 (222)	5.8 (59)	1.6 (16)	1.4 (14)
Immigrants					
Beijing	29	72.4 (21)	20.7 (6)	10.3 (3)	3.4 (1)
Non-Beijing	6	33.3 (2)	0 (0)	0 (0)	0 (0)
Overall					
Beijing	71	81.7 (58)	38 (27)	21.1 (15)	5.4 (4)
Non-Beijing	1024	21.9 (224)	5.8 (59)	1.6 (16)	1.4 (14)

rather than acquisition of MDR-TB during treatment that defines this association.

This is the first study on Beijing-TB in the patient population in Poland. Further molecular and epidemiological analyses are required to determine the genetic relatedness between strains and analyse possible transmission of tuberculosis between patients.

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