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Characterization and Mass Spectrometry Analysis of Aminopeptidase N from *Pseudomononas putida* Lup

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**Abstract**

An intracellular aminopeptidase N synthesized by *Pseudomonas putida* Lup was purified and characterized. The approx. 150-fold purified enzyme showed highest activity against A-β-naphthylamide at pH 7.5 and at temperature 40°C and was 100% thermostable for 240 min at 40°C. *P. putida* lup aminopeptidase N is a monomer with molecular mass approx. 99 kDa determined by SDS-PAGE and gel permeation chromatography. The enzyme has broad substrate specificity, but is the most active against protein substrates with N-terminal alanine and arginine. The activity of *P. putida* Lup aminopeptidase N is strongly inhibited in the presence of specific metallopeptidase inhibitors and is partly recovered in the presence of Zn²⁺ and Co²⁺ ions. Co²⁺, Mg²⁺ and Ca²⁺ ions increased the activity of the enzyme. Moreover, the enzyme was inhibited by inhibitors of cysteine enzymes. Analysis of fragments of the amino acid sequence of the purified enzyme demonstrated high similarity to PepN of *Pseudomonas putida* GB-1.

**Keywords:** *Pseudomonas putida*, aminopeptidase N, purification of enzymes

**Introduction**

Peptidases perform several significant functions in both eukaryotic and prokaryotic organisms. Intracellular peptidases are involved in processes such as splitting off signal peptides from newly synthesized proteins, activation of inactive precursors, inactivation of regulatory proteins and degradation of damaged peptides. Extracellular peptidases can be toxins and virulence factors, whereas others demonstrate low specificity and participate in the breakdown of proteins in the environment to short peptides or single amino acids, which can then be taken up and utilized by the cell (Addlagatta *et al.*, 2006; Jankiewicz and Bielawski, 2003). An important role in cellular protein metabolism in both eukaryotic and prokaryotic organisms is played by aminopeptidases N (APN, EC 3.4.11.2). APN found in living cells is a common exopeptidase with broad substrate specificity.

Mammalian aminopeptidases N are membrane enzymes that carry out diverse physiological functions, such as receptors for corona- and other human viruses. They also participate in angiogenesis and stimulation of tumor growth and regulation of blood pressure (Kumar *et al.*, 2009). The role of these exoproteases in physiological processes of the host justifies intensive studies on these enzymes and their inhibitors. In bacteria, aminopeptidases N occur in the cytosol where they participate in ATP-independent protein metabolism and play principal role in the maturation, activation and final stage of peptides hydrolysis to single amino acids (Addlagatta *et al.*, 2006; Lazdunski *et al.*, 1975). Bacterial APN in particular are the main intracellular enzymes releasing N-terminal alanine from protein substrates.

APN belong to the zinc-dependent metallopeptidases, grouped, according to the Merops classification, into family M1 included in the Gluzincin superfamily (thermolysin-like peptidases). All of these enzymes are metallopeptidases with a single zinc ion in the active center (Hooper, 1994; Rawlings and Barrett, 1993). APN have a characteristic conservative Zn-binding HEXXH(X)₆E motif, in which a Zn ion is coordinated by two histidine residues and distal glutamic acid. The glutamate next to the first Zn²⁺-binding histidine in the HEXXH motif is essential for water hydrolysis of
peptide bond and subsequent release of the substrate (Kumar et al., 2009; Peer, 2011). The structure of the active center has been studied in detail for the APN of the gram-negative bacterium Escherichia coli. It has been found that structurally it exhibits strong similarity to thermolysin. The APN of E. coli, which is composed of 870 amino acids, contains four domains, and the active center is located in domain II, encompassing amino acids residues from 194 to 443 (Matthews, 1988). There are also reports in the literature suggesting the presence of two active centers in APN, one responsible for aminopeptidase activity and one for endopeptidase activity (Chandu et al., 2003). However, more recent data rule out this hypothesis (Addlagatta et al., 2006). They are enzymes with broad substrate specificity, showing highest activity against protein substrates with the alkaline amino acid residues: R and K or hydrophobic ones, e.g. A, L, M, at the N-terminus (Chandu and Nandi, 2003; McCaman and Villarejo, 1982).

The objective of the described studies was to purify, identify and characterize the intracellular aminopeptidase synthesized by a strain of the bacterium Pseudomonas putida.

**Experimental**

**Materials and Methods**

**Biological materials.** A strain of bacteria synthesizing an intracellular aminopeptidase N (ApN) was isolated from the rhizosphere of winter wheat cultivated in a field in the eastern part of Poland. Bacteria were isolated using King B medium (King et al., 1954). Identification of the strain selected for further studies was based on biochemical and morphological traits (Holt et al., 1994). The identity of the strain was confirmed by 16S rRNA gene sequence analysis. Amplification of 16S rRNA gene was accomplished using universal primers 27 F and 1492 R (Watanabe et al., 2001). The template in the reaction was genomic DNA isolated from bacterial cells in late logarithmic stage of culture, using a Genomic DNA Purification Kit (Fermentas). The purified PCR product was sequenced in the DNA Sequencing and Oligonucleotides Synthesis Laboratory at IBB PAS (Warsaw, Poland). The obtained nucleotide sequences were analyzed with those deposited in the available databases GenBank/EMBL/DDBJ using the program BLAST. The obtained nucleotide sequences were deposited in GenBank/EMBL/DDBJ database with the accession number AB667905.

**Composition of culture medium, growth conditions and preparation of enzyme extract.** The liquid growth medium used was a mineral medium according to Bassalik, composed of: 0.03% K₂PO₄; 0.03% KH₂PO₄; 0.05% MgSO₄; 0.05% NaCl and 0.0015% FeCl₃, supplemented with 1.5% tryptone, 0.25% yeast extract and 0.3% glucose. The pH of the medium was adjusted prior to autoclaving to 7.0.

Bacteria were grown for 48 h with shaking at 120 rpm and temperature 28°C. Growth of bacteria was monitored by following OD₆₀₀. After appropriate culture time the bacteria were spun down (12000×g, 20 min) and the bacteria in the pellet, after discarding the supernatant, were washed twice in Tris-Cl buffer, pH 8.5 and disrupted by sonication. The clear intracellular protein extract obtained after centrifugation was used as the preparation for purification of the aminopeptidase.

**Enzymatic activity.** Aminopeptidase activity was determined using synthetic amino acid derivatives of β-naphthylamide. The incubation mixture consisted of: enzyme preparation, 0.05 M Tris-Cl buffer, pH 7.5 and substrate in final concentration 1 mM. The reaction was conducted for 30 minutes and then terminated by the addition of 0.1% solution of diazo salt of α-aminolucose (Fast Garnet GBC), dissolved in 1 M acetate buffer, pH 4.2 with the addition of 10% Tween 20. The absorbance of the colored product was determined photometrically at 525 nm. Absorbance readings were calculated to give μmole product formed using a standard curve prepared for five concentrations of β-naphthylamine.

One unit of activity was defined as one μmol naphthylamine formed in 1 min reaction time.

**Purification of the enzyme.** All purification stages (except for HPLC chromatography) were carried out at 4°C. Fractionation with ammonium sulfate was performed by salting out the enzyme preparation in the first stage of fractionation to 35%, and in the second to 85% saturation with ammonium sulfate. The protein deposit was dissolved in 20 mM Tris-Cl buffer, pH 7.8 and dialyzed overnight against the same buffer. The obtained preparation was subjected to low pressure ion-exchange chromatography on anionite cellulose DEAE 52.

Prior to chromatography, the column was equilibrated with 20 mM Tris-Cl buffer, pH 7.8. Protein was eluted with linear NaCl gradient from 0 to 0.5 M. Fractions with highest enzymatic activity were pooled and dialyzed for 12 hours against 20 mM Tris-Cl buffer, pH 7.8. The next stage of purification involved preparative electrophoresis in BIO-RAD Model 491 Prep Cell apparatus, with 7.5% separating gel and 4% concentrating gel, at constant voltage of 240 V. Separation was in 25 mM Tris – 192 mM glycine buffer, pH 8.3. Proteins were eluted from elution chamber with 25 mM Tris-Cl, pH 7.8. Fractions showing highest alanyl aminopeptidase activity were used for the next purification step, *i.e.* high pressure ion-exchange chromatography HPLC. The enzyme solution was applied to Protein-Pak Q 8HR column, which was first equilibrated with 15 mM Tris-Cl buffer, pH 7.8. Elution was with linear NaCl gradient from 0.2 to 0.5 M. Fractions with highest
Aminopeptidase N activity were used for enzyme characterization and MS analysis experiments.

**Determination of protein content.** Measurements of protein concentration at all stages of the study were made using the Bradford method (1976). Absorbance values were converted to µg protein using a standard curve plotted for five concentrations of bovine serum albumin.

**Electrophoretic separations and detection of aminopeptidase activity.** Electrophoresis under native and denaturing conditions was according to the procedure described by Laemmli (1970). The protein bands were visualized using Coomassie Brilliant Blue R-250. Aminopeptidase activity in polyacrylamide gel following electrophoretic separation was detected after incubation of the gel in 1 mM solution of the substrate in Tris-HCl buffer, pH 7.5.

**Determination of molecular weight of the enzyme.** The molecular weight of the analyzed enzyme was determined by SDS-PAGE and in the course of gel filtration on a Sephadex G-200 column. The column was calibrated using the following standard proteins: Alcohol dehydrogenase (150 kDa), bovine albumin (67 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa). The void volume of the column (Vo) was determined using dextran blue.

**Characterization of the properties of purified enzyme.** The optimum pH was determined in a range of 5.0–9.0. The buffer systems used were: 50 mM Britton-Robinson buffer for the pH range of 5.0–9.0 and 50 mM Tris-HCl buffer for the pH range of 6.8–8.5. The optimal temperature was determined in the range from 25 to 55°C. Thermal stability was determined after 1, 2, 3 and 4 h preincubation of the enzyme at temperatures: 40, 45 and 50°C.

**Determination of Michaelis-Menten constants.** The substrate affinities were determined in the presence of those substrates with which high enzymatic activity (over 60% compared to A-β-NA activity) was observed; the final concentration of the substrates in the reaction mix was from 0.015 to 1.5 mM. The Km value was determined using the graphical Lineweaver-Burk method.

The effect of metal ions on activity was determined following preincubation of the enzyme for 30 min at 4°C in the presence of divalent metal ions (Mg²⁺, Mn²⁺, Co²⁺, Ca²⁺, Cu²⁺, Zn²⁺, Cd²⁺, Pb²⁺) in final concentration of 0.1 and 1.0 mM, after which the substrate was added and the residual activity tested.

In order to study the effect of specific inhibitors on activity, the enzyme was preincubated with an inhibitor solution for 60 minutes at 4°C after which the remaining activity was tested.

Reactivation of protease activity after inhibition by EDTA was carried out in the presence of Ca, Mg and Zn ions following dialysis of the preparation.

**Analysis of fragment of the amino acid sequence of the purified enzymatic protein.** Both the highly purified enzyme preparation and the protein band (approx. 99 kDa) excised from polyacrylamide gel were subjected to mass spectrometry analysis (IBB PAN). The protein concentration in the sample was 10 µg/ml. A protein sample previously digested with trypsin was separated on a nanoAcquity UPLC (Ultra Performance LC) system and analyzed with an Orbitrap-based mass spectrometer.

All results presented in this paper in the form of numerical values are means from three independent repetitions. The mean error, reflecting maximal deviation of the results of measurements from the mean, did not exceed 5%.

**Abbreviations:**
- $K_m$ – Michaelis-Menten constant
- APN – Aminopeptidase N
- β-NA – β-naphthylamide
- EGTA – ethylene glycol tetraacetic acid
- EDTA – ethylenediaminetetraacetate

**Results**

The intracellular aminopeptidase synthesized by *Pseudomonas putida* Lup was purified over 150-fold in a four step procedure. The enzymatic activity of the enzyme during its purification and characterization was determined using A-β-NA as a substrate (Table I).

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total aminopeptidase activity [U]</th>
<th>Total protein [mg]</th>
<th>Specific activity [U/mg]</th>
<th>Fold</th>
<th>Yield purification [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>10227.3</td>
<td>1605.6</td>
<td>6.4</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation (35-85%)</td>
<td>8454.9</td>
<td>619.4</td>
<td>13.7</td>
<td>2.1</td>
<td>82.7</td>
</tr>
<tr>
<td>Ion-exchange chromatography cellulose DEAE 52</td>
<td>6715.4</td>
<td>186.6</td>
<td>36</td>
<td>5.6</td>
<td>65.7</td>
</tr>
<tr>
<td>Preparative electrophoresis</td>
<td>2312.5</td>
<td>7.9</td>
<td>292.7</td>
<td>45.7</td>
<td>20.6</td>
</tr>
<tr>
<td>Ion-exchange chromatography HPLC Mono Q 8HR</td>
<td>908.5</td>
<td>0.93</td>
<td>976.9</td>
<td>152.6</td>
<td>8.9</td>
</tr>
</tbody>
</table>
Salting out with ammonium sulfate and low pressure ion-exchange chromatography on DEAE cellulose resulted in a sample purified almost 6-fold with 65.7% recovery of total activity. Considerable, over 8-fold purification of the enzyme was obtained after preparative electrophoresis. After the final step of purification, ion-exchange HPLC chromatography, over 150-fold purification of the enzyme was achieved. The molecular weight of the enzyme determined by SDS PAGE was approx. 99 kDa (Fig. 1). This result was confirmed using molecular sieve chromatography, which determined a molecular weight of 98 kDa. This value shows that the studied enzyme is a monomer. Proteomic analysis of the purified enzymatic preparation using mass spectrometry (MS) demonstrated that the amino acid sequence of the studied enzymatic protein is highly similar to the sequence of the aminopeptidase N of *Pseudomonas putida* GB-1 (GenBank accession no. YP_001667790 which is identical to that translated from the nucleotide sequence of Gene ID: 58693290). The peptides derived from the studied aminopeptidase overlap with 45% of the aa sequence of aminopeptidase N from *P. putida* GB-1 (Fig. 2). High overlap of the obtained peptide sequence within the conserved domain of APN in *Pseudomonas* bacteria between amino acid residues 212 and 421 was determined. APN in strain GB-1 is composed of 885 amino acid residues. Its calculated molecular weight was 99.549 kDa and the theoretical pI was 5.06.

The purified *P. putida* Lup aminopeptidase has broad substrate specificity (Table II). The enzyme was shown to be active against the following amino acid derivatives of β-naphthylamide: A-β-NA (100%), R-β-NA (60%), K-β-NA (25%), L, S (10%) and G, M-β-NA (5%). The substrate affinity of *P. putida* lup aminopeptidase was determined for the substrates the enzyme had the highest activity against. Km constants determined did not show great variation, even though the lowest K_m was obtained during enzymatic reaction against A-β-NA, which points to the highest affinity of the enzyme for this particular substrate. The effect of specific inhibitors on the activity of the studied enzyme was also examined (Table III). Partial inhibition of enzyme activity observed when inhibitors such as EDTA and

---

Fig. 1. SDS-PAGE of the purified aminopeptidase.

Fig. 2. Sequence of aminopeptidase N from *P. putida* GB-1.

Sequence alignment of aminopeptidase N from *P. putida* GB-1 (GenBank accession no. YP_001667790 which is identical to that translated from the nucleotide sequence of Gene ID: 58693290) and studied aminopeptidase from *P. putida* lup (letters underlined).

Bold letters: Zn ion is coordinated by two histidine residues (H 303, 307) and distal glutaminic acid (326), the glutamate (304) is essential for water hydrolysis of peptide bond and subsequent release of the product.
Aminopeptidase N from *P. putida* 4

1.10 phenanthroline were added to the reaction mixture, it indicates that the protein is a metallopeptidase. In this case partial reactivation of activity was obtained after adding Co$^{2+}$ (20%) and Zn$^{2+}$ ions (50%). These results were confirmed in reaction with bestatin and amastatin, which are diagnostic inhibitors for metalloexopeptidases. However, inhibition of the enzyme was also demonstrated in the presence of cysteine protease inhibitors E64 and iodoacetamine. Moreover, after adding compounds counteracting the oxidation of -SH groups to the reaction mixture, such as reduced glutathione (GSH) or dithioerythritol (DTE), enhanced activity was observed, which is typical for cysteine enzymes (Table IV). Inhibitors specific for the remaining catalytic types of proteases (serine and aspartyl) did not cause any change in enzyme activity. The studied aminopeptidase was activated about 30–40% when Ca,

<table>
<thead>
<tr>
<th>Substrate</th>
<th>activity (%)</th>
<th>Km value [M]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-β-NA</td>
<td>100</td>
<td>6.8 × 10^{-5}</td>
</tr>
<tr>
<td>Arg-β-NA</td>
<td>60</td>
<td>7.8 × 10^{-5}</td>
</tr>
<tr>
<td>Lys-β-NA</td>
<td>25</td>
<td>7.5 × 10^{-5}</td>
</tr>
<tr>
<td>Leu-β-NA</td>
<td>10</td>
<td>nd</td>
</tr>
<tr>
<td>Ser-β-NA</td>
<td>10</td>
<td>nd</td>
</tr>
<tr>
<td>Gly-β-NA</td>
<td>5</td>
<td>nd</td>
</tr>
<tr>
<td>Met</td>
<td>5</td>
<td>nd</td>
</tr>
</tbody>
</table>

Not-determined-nd

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>85</td>
</tr>
<tr>
<td>1,10-Phenantroline</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>0.5</td>
<td>60</td>
</tr>
<tr>
<td>E-64</td>
<td>0.01</td>
<td>50</td>
</tr>
<tr>
<td>0.1</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>DFP</td>
<td>0.1</td>
<td>98</td>
</tr>
<tr>
<td>1</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Bestatin</td>
<td>0.0005</td>
<td>10</td>
</tr>
<tr>
<td>Amastatin</td>
<td>0.00005</td>
<td>0</td>
</tr>
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</table>

Table III
Effect of inhibitors on activity of *P. putida* Lup aminopeptidase.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Concentration (mM)</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>0.1</td>
<td>120</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>0.1</td>
<td>140</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>0.1</td>
<td>120</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>0.1</td>
<td>130</td>
</tr>
<tr>
<td>Pb$^{2+}$</td>
<td>0.1</td>
<td>115</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>0.1</td>
<td>130</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>Reduced glutathione</td>
<td>0.1</td>
<td>120</td>
</tr>
<tr>
<td>(GSH)</td>
<td>1.0</td>
<td>18</td>
</tr>
<tr>
<td>Dithioerythritol (DTE)</td>
<td>0.1</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>90</td>
</tr>
</tbody>
</table>

Table IV
Effect of metal ions and reducing agent on activity of *P. putida* Lup aminopeptidase.

![Fig. 3. Effect of pH on *P. putida* Lup aminopeptidase activity.](image-url)
Mg and Co ions in concentration 1.0 mM were added to them in the incubation mixture. Partial inhibition of activity was observed after the addition of Pb and Zn ions in low, 0.1 mM concentration (Table IV).

The temperature and pH optima determined for A-βNA were 40°C and pH 7.5, respectively. The studied aminopeptidase demonstrated high activity in a broad pH range from 6.0 to 8.5 (Fig. 3). On the other hand, the activity of the enzyme was strongly dependent on temperature and slight deviations from the optimal temperature resulted in its significant decrease (Fig. 4). The studied aminopeptidase was characterized by high thermal stability after 240 min preincubation at 40°C (Fig. 5). Preincubation for the same length of time at 45°C resulted in almost complete loss of activity.

Discussion

APN produced by *P. putida* belong to family M1, subfamily M01 and has been given the number 005. The conserved domain for family M1 stretches from amino acid residue 15 to 384, conserved residues for aminopeptidase N of Gram-negative bacteria – M01.005 are in the fragment from residue 212 to 421 (Hooper, 1994). So far the best characterized ApN of Gram-negative bacteria are those from *E. coli* (Ito et al., 2006) and *Neisseria meningitidis* (Nocek et al., 2008). The structure of these proteins has been investigated using crystallography methods, providing detailed information regarding their active center and the spatial structure of their functional domains. In the APN molecule from *E. coli*, four domains have been distinguished: N-terminal β-domain M'1-D193, catalytic domain: F194-G444, middle β-domain: T445-Y446 and C-terminal α-domain: S447-A470 (Addlagatta et al., 2006). The similarity of the amino acid sequence of aminopeptidases N of *E. coli* and *P. putida* is slightly over 50%.

There is far less information about the ApN of *Pseudomonas* sp. In case of *P. putida* ApN no detailed biochemical characterization of the enzyme has yet been conducted.

The aminopeptidase studied by our group has similar substrate specificity, except that its activity is the highest when the amino acid residue at the N-terminus of the protein substrate is alanine, and not arginine, like for the *E. coli* ApN. The substrate specificity of the *E. coli* ApN for N-terminal amino acids can be arranged as follows: A > R > K > P/G (Addlagatta et al., 2008). A somewhat different substrate specificity was found for aminopeptidase N of *Streptococcus thermophilus* A. The enzyme demonstrated highest activity towards the following substrates: K-AMC: 100% > L 93% > R: 80 > M: 28% > A: 20% (Chavagnat et al., 1999). Similar results were obtained also for *S. thermophilus* YRC001. For this reason the aminopeptidase N of these bacteria have been termed lysyl aminopeptidase (Motoshima et al., 2003). On the other hand, the N-like aminopeptidase of *Lactobacillus curvatus* DPC2024 showed highest activity with protein substrates carrying N-terminal L (100%), K (63%), F (58%), M (26%) and A (6%) (Abdallah et al., 1999). Studies on the APN of *E. coli* K12 have revealed that it is a metallopeptidase but a thiol group of a cysteine is also involved in catalysis.
(Yoshimoto et al., 1988). However, it has not yet been determined which of the 8 cysteine residues present in the APN sequence plays a crucial role for the activity of the enzyme (Nocek et al., 2008). It cannot be excluded that the binding of the inhibitor to one of the cysteine residues located close to the active site of APN results in distortion of its structure and changes in the conformation of the protein molecule. A similar phenomenon is observed for the studied ApN from P. putida lup. The enzyme is strongly inhibited by specific inhibitors of cysteine enzymes and metallopeptidases. Stimulation of enzyme activity in the presence of reducing compounds confirms the importance of the -SH groups of cysteine in enzymatic catalysis or in maintaining the proper conformation of the enzyme molecule. The amino acid sequence of the P.putida APN contains amino acids residues involved in the binding of zinc ions: H 303, H 307 and E 326. The significant role of zinc ions in catalysis is also supported by the partial reactivation by these ions of the studied aminopeptidase previously treated with EDTA. The studied aminopeptidase was activated in the presence of Co ions and to a somewhat lesser degree by Ca and Mg. A similar dependence was observed for the PepN-like enzyme in Lb. curvatus DPC2024 (Abdallah et al., 1999). Activation by Co ions has also been described for aminopeptidase N S. thermophilus YRC001 aminopeptidase (Motoshima et al., 2003).

The molecular mass of the studied enzyme determined by SDS PAGE is about 99 kDa. A similar molecular mass calculated based on the amino acid sequence of the genes coding the enzymes is given for other P. putida aminopeptidases N. Aminopeptidases N with similar molecular masses have also been found in E. coli K 12: 87 kDa (McCaman, and Villarejo, 1982) and Streptococcus thermophilus YRC001: 96.4 kDa (Motoshima et al., 2003). The pH and temperature optima for the studied enzyme are 7.5 and 40°C, respectively. APN of S. thermophilus A shows optimal activity under similar conditions: pH 7.0 and 37°C (Chavagnat et al., 1999). Similarly, the N-like aminopeptidase of Lh. curvatus demonstrates optimal activity at pH 7.0 and 40°C (Christensen et al., 1999).

Literature


**Production of Bacteriocin E50-52 by Small Ubiquitin-Related Modifier Fusion in Escherichia coli**

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**Abstract**

Bacteriocin E50-52, a class IIa bacteriocin with a wide antibacterial spectrum, and has a huge potential to be a substitute for conventional antibiotics. In this research, the bacteriocin E50-52 gene was cloned into the expression vector pET SUMO (small ubiquitin-related modifier) and introduced into *Escherichia coli* BL21 (DE3). The recombinant fusion protein SUMO-bacteriocin E50-52 expressed in a soluble form was purified to a purity of more than 90% by Ni-NTA sepharose column and 117 mg fusion protein was obtained per liter of fermentation culture. The fusion protein was cleaved with SUMO protease and re-applied to a Ni-NTA Sepharose column. Finally, about 16 mg recombinant bacteriocin E50-52 (rbE50-52) was obtained from a 1-liter fermentation culture with no less than 95% purity. The rbE50-52 had similar antimicrobial properties and molecular weight as the native bacteriocin E50-52 and showed very low hemolytic activity.

**Key words:** *Escherichia coli*, bacteriocin E50-52, purification, recombinant expression, small ubiquitin-related modifier

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**Introduction**

Bacteriocins are a family of ribosomally synthesized antimicrobial peptides produced by bacteria (Klaenhammer, 1993; Cleveland et al., 2001). Studies suggest that bacteriocins can inhibit bacteria by forming pores in the membrane and have effective activity against antibiotic-resistant pathogens (Jenssen et al., 2006; Svetoch and Stern, 2010). There is no report showing that bacteriocins could be toxic for animals and accumulate in the treated subjects (Svetoch and Stern, 2010). Therefore, as promising new biological products, bacteriocins could be developed into novel alternatives to traditional antibiotics (Joerger, 2003). Bacteriocin E50-52 is a class IIa bacteriocin produced by *Enterococcus faecium* NRRL B-30746 and has a molecular weight of 3339.7 Da. Bacteriocin E50-52 showed potent activity against both Gram-positive and Gram-negative bacteria in vitro tests and therapeutic trials. Moreover, bacteriocin E50-52 is heat-stable and can inhibit drug-resistant nosocomial bacteria effectively (Svetoch et al., 2008; 2009). Thus, bacteriocin E50-52 could be considered as a potential candidate for antibacterial therapy.

It is often cumbersome, time-consuming, and uneconomical to isolate bacteriocins from natural producer strains. Though chemical synthesis is a good way to obtain peptides of interest, it is limited by expensive reagents. Heterologous expression in *E. coli*, the first host used to produce a recombinant pharmaceutical peptide, is an adaptable method for the economical and rapid production of target proteins (Swartz, 2001; Li et al., 2007; Jasniweski et al., 2008). However, the significant antimicrobial activity of bacteriocins poses difficulties for direct expression in the host and high susceptibility to proteolytic degradation decreases the efficiency of purification (Li et al., 2009). For these reasons, the potential fusion expression strategy is employed to protect *E. coli* and target peptide (Rao et al., 2004; Wei et al., 2005; Morin et al., 2006; Shen et al., 2010; Ma et al., 2012).

SUMO is a member of the ubiquitin protein family that modulates the properties of intracellular proteins (Muller et al., 2001; Johnson, 2004). When fused with proteins, SUMO can fold and protect them via its chaperoning functions (Li et al., 2010). As a new fusion system, SUMO appears to have all the advantages of traditional systems and can be cleaved without extension in the N-terminus of the target proteins (Butt et al., 2005). Therefore, in this study, SUMO fusion protein expression system was used to produce rbE50-52.
Experimental

Materials and Methods

**Bacterial strains and growth conditions.** *E. coli* bacterial strain Mach1™-T1™ and strain BL21 (DE3) (Invitrogen, USA) were used as subcloning and expression host, respectively. Both *E. coli* Mach1™-T1™ cells and BL21 (DE3) cells were cultured and selected in LB broth or agar (1.8%) supplemented with kanamycin (50 μg/ml) at 37°C. *Staphylococcus aureus* ATCC 25923 was purchased from the China General Microbiological Culture Collection Center (Beijing, China), *E. coli* K88 and *Listeria monocytogenes* IVDCC 53005 were purchased from the China Veterinary Culture Collection Center (Beijing, China).

**Plasmid and gene sequence.** pET SUMO (Invitrogen) was used as expression plasmid. The GenBank accession number for the bacteriocin E50-52 used in this study is P85148.

**Construction of expression vectors.** The bacteriocin E50-52 gene was synthesized (Invitrogen) and amplified using primer pairs (Primer E50-52 F: 5'-ACCACCAAAAACATGGCCAAC-3'; Primer R: 5'-TCACTATTACGCCAGTTTGCACAG-3'). The resulting PCR product was separated by 1.8% gel electrophoresis, purified using a DNA gel extraction kit (Tiangen), and ligated into the linearized pET SUMO plasmid by TA cloning. The ligation mixture was transformed into *E. coli* Mach1™-T1™ cells for propagation of the recombinant plasmid. Another PCR was performed using primer pairs (Primer SUMO F: 5'-AGATTCCTTGTAAGCCAGGTATTAG-3'; Primer T7 R: 5'-TGAACACACAGTTTGCACAG-3'), and the product containing inserted fragment was sequenced to confirm insert orientation and sequence fidelity.

**Expression of rbE50-52 fusion protein.** The constructed pET SUMO-bacteriocin E50-52 plasmid was transformed into *E. coli* BL21 (DE3) strain. The recombinant expression strain was cultivated in LB broth containing kanamycin at 37°C with shaking (180 rpm) to an optical density (OD₆₀₀) of 0.7–0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 1.5 mM to induce the expression of the recombinant protein at 37°C for 5 h. To obtain the recombinant protein, the cells were treated according to the manufacturer’s instruction. The disrupted cells were centrifuged at 13,000 g for 2 min to separate the soluble supernatant and the insoluble pellet fraction. Both supernatant and precipitate were analyzed by Tricine-SDS-PAGE. The non-induced cells were used to a pre-equilibrated Ni-NTA (GE, USA) Sepharose column (5 ml), and the bound protein was eluted by elution buffer (50 mM NaPO₄ and 500 mM NaCl, 10–400 mM imidazole, pH 7.4) at 2 ml/min. The eluted samples were analyzed by Tricine-SDS-PAGE, and dialyzed against 150 mM NaCl overnight at 4°C.

**Cleavage and purification of rbE50-52.** The 500 μg fusion protein was treated with 250 U SUMO protease (Invitrogen) in 10 × SUMO protease buffer (2% Igepal (NP-40), 1.5 M NaCl, 10 mM DTT, 500 mM Tris-HCl, pH 8.0) 500 μl at 4°C for 15 h. After the cleavage, protein samples were subjected to a Ni-NTA Sepharose column to remove 6 × His-tagged carrier and undigested fusion proteins. The purified rbE50-52 was analyzed by Tricine-SDS-PAGE.

**Determination of the molecular weight by MS.** MS analysis was carried out on AB4700 MALDI-TOF/TOF MS (AB Company, USA). Lyophilized rbE50-52 was dissolved with solvent A (70% acetonitrile, 0.1% trifluoroacetic acid in water [v/v/v]), and 0.5 μl samples mixed directly on the target with 1.5 μl of matrix (20 μg of α-cycno-β-hydroxy-cinnamic acid/μl in solvent A). The collected data were analyzed using Data Explorer V4.5 software (Applied Biosystems, USA).

**Antimicrobial activity assay.** The antimicrobial activity of rbE50-52 was tested by the agar diffusion method (cylinder plate method) using *S. aureus* ATCC 25923 as indicator strain. Cylinders were placed on the surface of agar inoculated with the test organism (about 1 × 10⁶ CFU/ml) and filled with 200 μl of purified rbE50-52 (16 μg/ml). The same volume of sodium phosphate buffer (PBS) and SUMO-bacteriocin E50-52 fusion protein were used as negative control. After incubation overnight at 37°C, zones of growth inhibition surrounding the cylinders were measured. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by a liquid growth inhibition test using *S. aureus* ATCC 25923, *E. coli* K88 and *L. monocytogenes* IVDCC C53005 as indicator strains as described by Karthikeyan (2006).

**Hemolytic assay.** The hemolytic property of rbE50-52 was evaluated by examining hemoglobin release at 540 nm in suspensions of rabbit erythrocytes. Bacteriocin at different concentrations (5, 10, 25, 50, 100 μg/ml) was used in this experiment. The fresh rabbit blood was treated according to the method of Tian (2009). The absorption was recorded at 540 nm using Imark™ Microplate Reader (Bio-Rad, US). The values for 0% and 100% hemolysis were determined in 10 mM PBS and 0.1% Triton X-100, respectively.

**Results**

**Construction of expression vectors.** The schematic representation of the bacteriocin E50-52 expression vector, containing a His-tag for affinity purification, is
Production of bacteriocin in *E. coli* presented in Fig. 1. The correct orientation of the insert was confirmed by PCR amplification and sequencing (data not shown).

**Expression of the fusion protein.** *E. coli* BL21 (DE3) was transformed with pET SUMO-bacteriocin E50-52 plasmid and induced as described above. After disruption by sonication, supernatant and precipitate were subject to Tricine-SDS-PAGE. As shown in Fig. 2, there was an obvious protein band after IPTG induction compared with non-induced cells. The result indicated that the fusion protein was efficiently produced in a soluble form in the host induced by 1.5 mM IPTG at 37°C.

**Purification of fusion protein.** The fusion protein contains polyhistidine (6×His) tag at N-terminus and can be separated efficiently using a Ni-NTA Sepharose column. Most of the proteins without His-tag were washed with washing buffer (the concentration of imidazole < 300 mM), and the 6×His-tagged SUMO-bacteriocin E50-52 was eluted by the elution buffer (50 mM Na<sub>3</sub>PO<sub>4</sub>, 500 mM NaCl, 300 mM Imidazole, pH 7.4) with more than 90% purity (Fig. 3). About 117 mg fusion protein can be obtained from 1-liter of fermentation culture.
Cleavage and purification of rbE50-52. Purified SUMO-bacteriocin E50-52 fusion protein was cleaved using SUMO protease to release the rbE50-52, and then confirmed by Tricine-SDS-PAGE. Subsequently, the mixture was loaded to a Ni-NTA Sepharose column and rbE50-52 was washed with washing buffer. As shown in Fig. 4, the fusion protein was successfully cleaved (lane 2) and rbE50-52 was purified efficiently (lane 3). About 16 mg of purified mature rbE50-52 with more than 95% purity was obtained from 1-liter fermentation culture.

Determination of the molecular weight. The precise molecular weight of rbE50-52 determined by MS was 3339.72 Da, which is in good agreement with that of the native bacteriocin E50-52 (Fig. 5). The result suggested that the bacteriocin E50-52 was expressed correctly and the fusion protein was cleaved at expected position with no extra amino acid in the N-terminus of recombinant peptide.

Antimicrobial activities of rbE50-52. The rbE50-52 showed strong inhibitory activity against S. aureus ATCC 25923 (Fig. 6). The MIC and MBC against selected strains are presented in Table I. The results indicated that the rbE50-52 had similar antibacterial properties against indicator bacteria as the native bacteriocin E50-52 and was effective in killing bacteria.

Hemolytic activity. The release of hemoglobin was used to evaluate the hemolytic activity of rbE50-52. The hemolysis percentages of the peptide at concentration of 5, 10, 25, 50, 100 μg/ml were 0.76 ± 0.65%, 0.85 ± 0.57%, 1.98 ± 0.67%, 2.11 ± 0.49%, 2.87 ± 0.76%, respectively.

**Table 1**
The MIC/MBC of rbE50-52 and native bacteriocin E50-52 to selected microorganisms

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC/MBC (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>rbE50-52</td>
</tr>
<tr>
<td>E. coli K88</td>
<td>0.9/1.7</td>
</tr>
<tr>
<td>S. aureus ATCC 25923</td>
<td>0.5/2</td>
</tr>
<tr>
<td>L. monocytogenes IVDC 53005</td>
<td>14/32</td>
</tr>
</tbody>
</table>

* The MIC of the native bacteriocin E50-52 against E. coli, S. aureus and L. monocytogenes were determined using many different isolates.

b The MBC of native bacteriocin E50-52 against selected bacteria was not reported.

Discussion

Drug resistance among variety of microorganisms poses a potentially serious threat to public health and has caused substantial alarm and concern (Svetoch et al., 2008; 2009). It becomes urgent to develop antimicrobial compounds with new mechanisms of action and potent activity against these microorganisms. Bacteriocins can inhibit bacterial growth by disruption of target cell membrane and have a low frequency of bacteriocin-resistant mutants (Sang and Blecha, 2008; Svetoch and Stern, 2010). Bacteriocin E50-52 displayed strong inhibitory property against both Gram-positive and Gram-negative bacteria and was also effective against antibiotic-resistant bacteria isolated from hospitals in vitro test (Svetoch et al., 2008; 2009). In vivo test in broiler, oral treatment with bacteriocin E50-52 reduced both Salmonella enteritidis and Campylobacter jejuni by more than 100 000-fold in the ceca, and systemic S. enteritidis was reduced in the liver and spleen (Svetoch et al., 2008). In this work, the MBC and hemolytic activity of rbE50-52 were also measured.
peptide showed effective bactericidal properties against indicator strains and produced very low hemolysis against rabbit red blood cells. These characteristics of bacteriocin E50-52 make it potentially valuable as an agent for antimicrobial chemotherapy.

The low efficiency of bacteriocin purification from natural producer strains and high cost of chemical synthesis led to the exploration of heterologous expression of peptides with antimicrobial property against bacteria (Li et al., 2010). As one of the most commonly used heterologous expression host, E. coli was utilized to produce rbE50-52 in this study. Due to their high toxicity to the expression host cells and sensitivity to proteases, direct expression systems of recombinant bacteriocins are difficult to be established. So far, the best available tools for expression of these proteins are fusion protein partners, which can also increase expression level, enhance protein solubility, and simplify purification and detection of recombinant proteins (Butt et al., 2005; Esposito and Chatterjee, 2006; Li, 2009; Sun et al., 2008). Today, a number of fusion protein partners are used in the expression of proteins, such as MBP (maltose-binding protein), GST (glutathione S-transferase), TRX (thioredoxin), Nus A, Ub (ubiquitin) and SUMO. Comparative study showed that SUMO was superior to commonly used fusion partners (Marblestone et al., 2006). SUMO proteases are able to cleave SUMO tag accurately and release the recombinant protein with native N-terminus, which is prerequisite for maintaining the activity of bacteriocins (Wu and Hancock, 1999; Butt et al., 2005). In the current study, the fusion protein was expressed and purified efficiently with the aid of SUMO fusion protein partners. After cleavage, the rbE50-52 with desired N-terminus was released. The recombinant peptides had similar antimicrobial properties (MIC) and molecular weight as the native bacteriocin E50-52. About 16 mg rbE50-52 was obtained from one liter of fermentation culture, the yield of native E50-52 was not reported. Compared with isolating from natural producer strains, producing of rbE50-52 needed cheaper medium, shorter fermentation time and simpler purification procedures (Svetoch et al., 2009).

In summary, we constructed an efficient SUMO fusion system to express and purify E50-52 in E. coli. The system can produce biologically active bacteriocin E50-52 with acceptable yield, and the purification is achieved in two steps. This expression system can potentially be a powerful tool for obtaining sufficient quantities of bacteriocin E50-52 for further studies or possible application.

Acknowledgements

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Literature


Introduction

Acidithiobacillus caldus (A. caldus) is a Gram-negative, strictly aerobic, rod-shaped bacterium which derives energy from the oxidation of reduced sulfur compounds. These bacteria appear to be found in acid mine drainage (AMD) and bioleaching reactors in moderately thermophilic environments (Baker and Banfield, 2003; Johnson and Hallberg, 2003; Zhou et al., 2009). It is considered to be the dominant sulfur-oxidizing bacterium in the bioleaching of sulfide ores at 40°C–50°C (Foucher et al., 2003; Okibe et al., 2003).

The main roles of A. caldus in bioleaching processes include: 1) removing the accumulated elemental sulfur on the surface that would otherwise retard the oxidation of ores. 2) producing organic growth factors that may stimulate heterotrophic and mixotrophic growth of bacteria. 3) producing surface-active agents to solubilize the S0 (Dopson and Lindström, 1999; Watling, 2006).

In China, bacterial oxidation has been used to recover many metals such as gold, copper, uranium, nickel and manganese from ores. And applications of the bio-oxidation of copper, gold and uranium have been commercialized successfully. In order to control and optimize metal bioleaching, some quick and reliable methods are needed to identify and quantify single species in complex bioleaching communities. Various molecular methods, such as 16S rRNA gene sequence analysis, DNA G+C content, DNA-DNA hybridization, DGGE (denaturing gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism), RISA (ribosomal intergenic spacer analysis), PFGE (pulsed-field gel electrophoresis), RAPD (random amplified polymorphic DNA), rep-PCR (repetitive extragenic palindromic polymerase chain reaction), FISH (fluorescent in situ hybridization) and real-time PCR, have been used to obtain a better understanding of the phylogenetic relationships of the interspecific and intraspecific strains (Mohapatra et al., 2011). These molecular typing techniques can generate differential DNA fingerprinting based on different principals, and hence differentiate different microorganisms.

However, little is known about the diversity of A. caldus strains involved in the bacterial oxidizing process.

Comparative Study of PCR-Based Approaches for the Genetic Characterization of Three Strains of Acidithiobacillus caldus Isolated from Different Sites in China

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Abstract

Comparative study of the genetic characteristics among three Acidithiobacillus caldus strains isolated from different typical environments in China was performed using a combination of molecular methods, namely sequencing analysis of PCR-amplified 16S rRNA genes and 16S-23S rRNA gene intergenic spacers (ITS), repetitive element PCR (rep-PCR), arbitrarily primed PCR (AP-PCR) fingerprinting and random amplified polymorphic DNA (RAPD). Both of the 16S rRNA gene and 16S-23S rRNA gene intergenic spacers sequences of the three strains exhibited small variations, with 99.9–100%, 99.7–100% identity respectively. In contrast, according to the analysis of bacterial diversity based on rep-PCR and AP-PCR fingerprinting, they produced highly discriminatory banding patterns, and the similarity values between them varied from 61.97% to 71.64%. RAPD analysis showed that banding profiles of their genomic DNA exhibited obvious differences from each other with 53.44–75% similarity. These results suggested that in contrast to 16S rRNA genes and 16S-23S rRNA gene intergenic spacers sequencing analysis, rep-PCR, AP-PCR fingerprinting and RAPD analysis possessed higher discriminatory power in identifying these closely related strains. And they could be used as rapid and highly discriminatory typing techniques in studying bacterial diversity, especially in differentiating bacteria within Acidithiobacillus caldus.

Keywords: Acidithiobacillus caldus, 16S rRNA gene, AP-PCR and Rep-PCR, ITS, RAPD

Introduction

Acidithiobacillus caldus (A. caldus) is a Gram-negative, strictly aerobic, rod-shaped bacterium which derives energy from the oxidation of reduced sulfur compounds. These bacteria appear to be found in acid mine drainage (AMD) and bioleaching reactors in moderately thermophilic environments (Baker and Banfield, 2003; Johnson and Hallberg, 2003; Zhou et al., 2009). It is considered to be the dominant sulfur-oxidizing bacterium in the bioleaching of sulfide ores at 40°C–50°C (Foucher et al., 2003; Okibe et al., 2003). The main roles of A. caldus in bioleaching processes include: 1) removing the accumulated elemental sulfur on the surface that would otherwise retard the oxidation of ores. 2) producing organic growth factors that may stimulate heterotrophic and mixotrophic growth of bacteria. 3) producing surface-active agents to solubilize the S0 (Dopson and Lindström, 1999; Watling, 2006).

In China, bacterial oxidation has been used to recover many metals such as gold, copper, uranium, nickel and manganese from ores. And applications of the bio-oxidation of copper, gold and uranium have been commercialized successfully. In order to control and optimize metal bioleaching, some quick and reliable methods are needed to identify and quantify single species in complex bioleaching communities. Various molecular methods, such as 16S rRNA gene sequence analysis, DNA G+C content, DNA-DNA hybridization, DGGE (denaturing gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism), RISA (ribosomal intergenic spacer analysis), PFGE (pulsed-field gel electrophoresis), RAPD (random amplification of polymorphic DNA), rep-PCR (repetitive extragenic palindromic polymerase chain reaction), FISH (fluorescent in situ hybridization) and real-time PCR, have been used to obtain a better understanding of the phylogenetic relationships of the interspecific and intraspecific strains (Mohapatra et al., 2011). These molecular typing techniques can generate differential DNA fingerprinting based on different principals, and hence differentiate different microorganisms.

However, little is known about the diversity of A. caldus strains involved in the bacterial oxidizing process.
The aim of the present study was to characterize the genomic heterogeneity of *A. caldus* strains, using a combination of molecular systematic methods, namely sequencing analysis of PCR-amplified 16S rRNA genes and 16S–23S rRNA gene intergenic spacers (ITS), repetitive element PCR (rep-PCR), arbitrarily primed PCR (AP-PCR) fingerprinting and random amplified polymorphic DNA (RAPD). In addition, this will provide useful information for researchers interested in microbial ecology and bioleaching as it not only describes the diversity of *A. caldus* within the species level systematically, which is rare in diversity research, but also targets on the dominant sulfur-oxidizing bacterium in the bioleaching of sulfide ores at 40°C–50°C.

**Experimental**

**Materials and Methods**

**Bacterial strains, media and culture conditions.** This study was conducted with three strains of *A. caldus* recovered from two main geographical locations: acid mine drainage site and coal heap site. Among them, the *A. caldus* strains S-1, S-2 were isolated from samples from the coal heap drainage in Changsha, Hunan Province, China. While *A. caldus* strain D-1 was isolated from AMD of Dabaoshan Mineral Company in Guangdong Province, China. All of the *A. caldus* strains were cultured using the typical 9K medium ([(NH₄)₂SO₄ 3.0 g/l, KCl 0.1 g/l, K₂HPO₄ 0.5 g/l, MgSO₄·7H₂O 0.5 g/l, Ca(NO₃)₂ 0.01 g/l)] with S⁰ (10 g/l) and pH adjusted to 2.5 under the temperature of 45°C.

**DNA preparation.** Bacterial cells were harvested by centrifugation at 7378 × g for 10 min and genomic DNAs of all bacteria were extracted using the TIANamp Bacteria DNA Kit (Tiangen Corporation Ltd. Beijing, China). DNA quantity was determined by spectrophotometry (Nanodrop Technologies, Rockland, DE). All DNAs were stored at −20°C until being used.

**Random amplified polymorphic DNA (RAPD).** Twenty 10-mer primers from Operon Technologies Inc. (Alameda, CA, USA) were used for the PCR reactions (Table I). PCR reaction mixtures (25 µl) contained 1 µl (30 ng) of DNA, 2 µl (10 µM) of the primer, 2.5 units (0.5 µl) of Taq DNA Polymerase (Tiangen Corporation Ltd. Beijing, China), 2.5 µl of 10 × Taq buffer, 2 µl (2.5 mM) dNTP Mixture and 17 µl ddH₂O. PCR reaction was performed in a thermocycler (Applied Biosystems Corporation, Ltd., USA). The amplification conditions were: an initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPC-01</td>
<td>TTTGGGTAGGGC</td>
</tr>
<tr>
<td>OPC-02</td>
<td>CAGCGCTCAAG</td>
</tr>
<tr>
<td>OPC-03</td>
<td>TGGATACCTGCC</td>
</tr>
<tr>
<td>OPC-04</td>
<td>GCCGATCGCA</td>
</tr>
<tr>
<td>OPC-05</td>
<td>GTAACGGGAT</td>
</tr>
<tr>
<td>OPC-06</td>
<td>GAGGGGCTTC</td>
</tr>
<tr>
<td>OPC-07</td>
<td>GATCGGATC</td>
</tr>
<tr>
<td>OPC-08</td>
<td>GTCACCGG</td>
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<td>OPC-09</td>
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<td>OPC-10</td>
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<tr>
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<td>OPC-12</td>
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<td>OPC-13</td>
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<td>CACACTCCCG</td>
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<td>TGAGTGGGT</td>
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<td>GCTGCGCCG</td>
</tr>
<tr>
<td>OPC-20</td>
<td>ACTGCAGCC</td>
</tr>
</tbody>
</table>

Each amplification was done in triplicate or more and results were reproducible when DNA from different extractions of the same strain were used (Bergamo et al., 2004; Novo et al., 1996). The amplification products (10 µl) were separated by electrophoresis on 1.5% agarose gel using 1 × TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1.8 mM EDTA, pH adjusted to 7.8 with glacial acetic acid) for 3 h at 40 V. The gels were stained with ethidium bromide and photographed under UV. The 200 bp DNA ladder (Tiangen Corporation Ltd. Beijing, China) was used as a size marker in all gels. The results were analyzed by comparing RAPD profiles on the basis of the presence (1) or absence (0) of each DNA band on the photographed agarose gels. Similarity analysis was performed using the DICE similarity coefficient in the NTSYS-PC software (Campos Paulino et al., 2001).

**Rep-P CR and AP-P CR amplification.** All PCR reactions were performed in a total volume of 25 µl using 30–50 ng DNA, and carried out in duplicate using a thermocycler (Applied Biosystems Corporation, Ltd., USA). BOX-PCR (based on primers targeting the highly conserved repetitive DNA sequences of the BOXA subunit of the BOX element) and ERIC-PCR (based on primers targeting the highly conserved enterobacterial repetitive intergenic consensus) were conducted to obtain the genomic fingerprinting of the bacteria described above. The rep primers were: for ERIC-PCR, ERIC-1R: 5’-ATGTAAGCTCCTGGGGATTCAC-3’, ERIC-2: 5’-AAGTAAGTCGACTGGGTTAGG-3’, and
for BOX-PCR, BOXA1R: 5'-CTACGGCAAGGCCGAGCGTTGAGC-3' (Versalovic et al., 1991; Versalovic et al., 1994). The reaction mixtures (25 µl) contained 2.5 µl (10 µM) of each primer and 5 µl of BOX primer for BOX-PCR, 2.5 units (0.5 µl) of Taq DNA Polymerase (Tiangen Corporation Ltd. Beijing, China), 2.5 µl of 10 × Taq buffer, 2 µl (2.5 mM) dNTP Mixture, 1 µl DNA and 14 µl ddH₂O. The amplification conditions included an initial denaturation at 95°C for 7 min, followed by 40 cycles of denaturation at 90°C for 30 s, annealing at either 55°C (BOX-PCR) or 52°C (ERIC-PCR) for 30 s, and amplification at 72°C for 8 min, followed by a final extension at 72°C for 10 min. PCR products were then examined through horizontal electrophoresis in 2% agarose gel containing ethidium bromide at 60 V for 5 h in 1 × TAE buffer and photographed under UV.

In the AP-PCR amplification, primers M13 (5'-TTATGTAATACGACGGCCAGC-3'), (Jonas et al., 2000), BG2 (5'-TACATTCGAGGACCCCTAAGTG-3') (Van Belkum et al., 1993) and PJ118 (5'-TGTTCGT-GCTTGTTCTG-3'), PJ108 (5'-GCTATTCTTGA-CATCCA-3') (Lado and Yousef, 2003) were used. The PCR reaction mixtures (25 µl) contained 1 µl 30 ng of DNA, 2.1 (10 µM) of the primer, 2.5 units (0.5 µl) of Taq DNA Polymerase (Tiangen Corporation Ltd. Beijing, China), 2.5 µl of 10 × Taq buffer, 2 µl (2.5 mM) dNTP Mixture and 17 µl ddH₂O. PCR was initiated with two cycles of low stringency, which included a denaturing step at 95°C for 1 min, annealing of the primer at 36°C for 1 min, and 2 min of extension at 72°C. After the initial two cycles, 40 additional cycles were conducted with annealing of the primer at 55°C. The reaction was terminated with a final extension cycle at 72°C for 10 min. The amplification products (12.5 µl) were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide at 60 V for 5 h in 1 × TAE buffer and photographed under UV.

Results

Analysis based on RAPD-PCR data. The genomic DNA of each strain used in this work was amplified using 20 different random primers. Eighteen of the primers tested produced a large number of specific and reproducible banding patterns revealing polymorphisms, whereas the other two primers (OPC-10, OPC-14) were excluded from the data analysis due to the production of non-reproducible banding pattern. The size of the amplified products ranged from approximately 200 to 3400 bp. The RAPD profiles of the three A. caldus strains were compared, and variation in the band profiles was observed for most primers (Fig. 1 presents only PCR results with primers OPC-5 and OPC-13). The similarity ratio among strains was calculated from the genomic DNA banding profiles generated by RAPD experiments. As shown in Table II, the highest similarity ratio of genomic DNA is between D-1 and S-1 (75%), and the next is between S-2 and S-1 (59.26%). The lowest similarity ratio is between S-2 and D-1 (53.44%).
Analysis of bacterial diversity based on rep-PCR and AP-PCR fingerprinting. Rep-PCR and AP-PCR experiments were performed to investigate the genotypic heterogeneity of the tested strains using the set of rep primers (BOX and ERIC) and AP primers (M13, PJ108, PJ118 and BG2). The results showed that the DNA banding profiles of the three strains exhibited obvious differences from each other. BOX-PCR yielded fingerprints with bands ranging from approximately 250 to 2000 bp, and the ERIC profiles encompassed bands ranging from approximately 200 to 2500 bp, whereas AP-PCR generated DNA fragments ranging in size from approximately 200 to 2100 bp. Almost every isolate yielded a unique and complex genomic fingerprint with each primer (Fig. 2 presents the results of ERIC-PCR (a) and AP-PCR results with M13 primer (b)). The similarity ratio among them was calculated from the genomic DNA banding profiles generated by rep-PCR and AP-PCR experiments. As shown in Table II, the highest similarity ratio of genomic DNA is between S-2 and S-1 (71.64%), and the next is between S-1 and D-1 (68.57%). The lowest similarity ratio is between S-2 and D-1 (61.97%).

Comparative analysis of 16S rRNA genes and 16S-23S rRNA gene intergenic spacers sequences. Amplifications and sequences of the 16S rRNA genes were carried out with genomic DNA from the three *A. caldus* strains recovered from acid mine drainage or coal heap sites in China. According to our sequencing results, the nucleotide sequences of 16S rRNA gene from these
The genetic characteristics of *Acidithiobacillus caldus*

Three strains were 99.5–100% identical to that of the *A. caldus* strain DSM 8584\textsuperscript{T} in GenBank (Z29975). The 16S rRNA gene sequences of D-1 and S-2 were completely identical, while the 16S rRNA gene sequence of S-1 was somewhat different from that of the other two strains with small variation. The similarity ratios among them calculated from the sequences of 16S rRNA gene were shown in Table III. Consequently, the variations of the 16S rRNA gene sequences are not sufficient to differentiate some closely related *A. caldus* strains.

At the same time, we have also determined the diversity of 16S-23S rRNA gene intergenic spacers among the tested *A. caldus* strains. According to our sequencing results, the structure and organization of the 16S-23S rRNA gene intergenic spacers of the Chinese *A. caldus* isolates were consistent with the data of the type strain *A. caldus* DSM 8584\textsuperscript{T} (Table IV). The 16S-23S rRNA gene intergenic spacers of all *A. caldus* isolates contain two highly conserved genes for tRNA\textsuperscript{Ile} and tRNA\textsuperscript{Ala}, which split the ITS into three regions (ITS1, ITS2 and ITS3). Sequencing analysis showed that the nucleotide sequences of 16S-23S rRNA gene intergenic spacers from these three strains were 99.7–100% identical to that of the *A. caldus* strain DSM 8584\textsuperscript{T} in GenBank (AF512808). The nucleotide sequences of the 16S-23S rRNA gene intergenic spacers from S-1 and D-1 were completely identical to each other, while the 16S-23S rRNA gene intergenic spacers sequence of S-2 was somewhat different from that of the other two strains with only one nucleotide change (nucleotide 262, C→T, Fig. 3). The similarity ratios among them calculated from the sequences of 16S-23S rRNA gene intergenic spacers are shown in Table III.

**Discussion**

Investigations have demonstrated that varied growth conditions, such as degree of pollution, substrate type and strength of toxic metal ions, can influence the structural changes in chromosomal DNA of *Acidithiobacillus* strains reflecting the genetic adaptation of the different ecotypes of *Acidithiobacillus* strains to different natural environments (Kondratyeva *et al.*, 1995; Tupikina *et al.*, 2003).
Therefore, natural selection and genetic adaptation may be the reason why *A. caldus* strains occupying specific microbial niches in diverse environments are heterogeneous from each other.

In addition, previous studies have also demonstrated that most *Acidithiobacillus* strains isolated from various parts of the world contain a variety of insert sequences (IS) involved in the adaptation to changes in environmental conditions and in the regulation of the expression of biochemical pathways, which in turn result in changes to some phenotypic traits, including resistance to metal ions and the capacity for substrate oxidation (Rawlings, 2001; Kondrat’ eva et al., 2005). Therefore, these mobile IS elements may be another cause resulting in the phenotypical and genotypical heterogeneity of *Acidithiobacillus* strains.

In the present work, the genomic heterogeneity of three *A. caldus* strains were analyzed and compared using a combination of molecular systematic methods, namely sequencing analysis of PCR-amplified 16S rRNA genes and 16S-23S rRNA gene intergenic spacers, repetitive element PCR, arbitrarily primed PCR fingerprinting and random amplified polymorphic DNA. Recently, polymorphism analysis of 16S rRNA genes and 16S-23S rRNA gene intergenic spacers has been used extensively to identify the diversity analysis of microorganisms at the species level or above due to their high conservation (Gürtler and Stanisich, 1996; Gomes et al., 2010; Breuker et al., 2009). In addition, some studies have showed that in contrast to the 16S rRNA gene, polymorphism analysis of 16S-23S rRNA gene intergenic spacers exhibits considerable variation in structure and is more appropriate for studies at the intraspecies level (Ni et al., 2007; Darwish and Ismaiel, 2005). As described in this study, however, *A. caldus* strains from different econiches possessed only small variations in both with about 99.9–100% identity of 16S rRNA gene, 99.7–100% identity of 16S-23S rRNA gene intergenic spacers respectively. It can be, therefore, suggested that some closely related strains may not be distinguishable on the 16S rRNA gene and 16S-23S rRNA gene intergenic spacers level.

Rep-PCR and AP-PCR fingerprinting have been widely used to detect polymorphism in bacteria including *Salmonella*, *Arthrobacter*, *Rhodococcus*, and *Pseudomonas* (Liang et al., 2010; Albufera et al., 2009; Boulygina et al., 2009). They can differ in the rep-PCR and AP-PCR profiles which might lead to detect interspecies and intraspecies differences based on agarose patterns (De Meyer et al., 2011; Prieto et al., 2007; Emekdas et al., 2006). As described in this study, in the analysis of bacterial diversity based on rep-PCR and AP-PCR fingerprinting, almost every of the strains yielded a unique and complex genomic fingerprint with each primer. The three *A. caldus* strains exhibited obvious differences from each other with 61.97–71.64% identity. These results, as well as others, clearly indicated that rep-PCR combined with AP-PCR fingerprinting could
discriminate and differentiate closely related strains fastly and successfully (Ni *et al*., 2008; Xie *et al*., 2008; Ranjbar *et al*., 2007). Therefore rep-PCR combined with AP-PCR may be used as a quick and reliable alternative for establishing differences among strains of the same species as well as among different species.

Likewise, RAPD analysis has been widely used to differentiate bacterial strains among diverse species including *Lactobacillus, Leuconostoc,* and *Acidithiobacillus* (Omar *et al*., 2000; Ni *et al*., 2008; Abed and Has- san, 2009), and it has also been shown to be a powerful technique in detecting genomic diversity on the strain level (Martinez-Blanch *et al*., 2011; Waltenbury *et al*., 2005). In this study, RAPD analysis further showed that the genomic DNA banding profiles of the three strains exhibited obvious differences from each other, and the similarity between the three strains obtained in the RAPD patterns analysis ranged from 53.44% to 75%. These results are in agreement with previous studies which reported that the similarity coefficients within *A. ferrooxidans* strains ranging from almost 0% to over 98% (Novo *et al*., 1996). This study further proves that RAPD is a sensitive technique to assess strain variability among the same species (Waltenbury *et al*., 2005; Akbar *et al*., 2005).

In conclusion, in our study, *A. caldus* strains from different sources with a high degree of similarity 16S rRNA gene and 16S–23S rRNA gene intergenic spacers sequences, could be distinguished on the basis of rep-PCR, AP-PCR fingerprinting and RAPD patterns. Therefore, rep-PCR, AP-PCR fingerprinting and RAPD-PCR profiles revealed a high degree of genomic diversity within *A. caldus* strains, and these methods could become important tools in the classification and identification of these bacteria. However, the accurate and reliable monitoring the genomic heterogeneity of microbes can only be achieved by integration of different molecular typing methods.

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**Literature**


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ORIGINAL PAPER

Biological Evaluation of Quaternary bis Ammonium Salt and Cetylpyridinium Bromide Against S. epidermidis Biofilm

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Abstract
Quaternary ammonium compounds are broad-spectrum bacteriocides widely used as antiseptics, disinfection and preservation agents. The aim of this study was to examine the activity of two quaternary ammonium salts, cetylpyridinium bromide and a newly synthesized quaternary bis ammonium salt, against S. epidermidis biofilm. The average values of killing efficiency for cetylpyridinium bromide ranged from 26.6% to 64.1% for all tested concentrations (0.125 to 8.0 µg×mL–1) and for quaternary bis ammonium salt the percentage of killing efficiency ranged from 59.7% to 88.4% for tested concentrations (from 2.0 to 128.0 µg × mL–1). Both tested compounds significantly affect staphylococcal biofilms, but any of used concentrations caused a total eradication of bacterial biofilm.

Key words: bacterial biofilm, Staphylococcus epidermidis, quaternary ammonium compounds

Introduction
Many strains of Gram-positive bacteria are dangerous pathogens causing severe hospital infections. Apart from infections of clean wounds, the special attention should be paid to infections occurring in patients who have received implants, i.e. valves, vascular and bone implants, joint prostheses, or tendon prostheses made of biomaterials (Wójcikowka-Mach et al., 2002; Christensen, 1982; Götz, 2002; Mack et al., 2006). Due to the incorrect use of antiseptics before and after surgery, colonisation of the surgical wound by the microbes originating from the hospital environment, occurs quite frequently. Microbes also can be transmitted from the hands of the medical personnel, or transferred from the patient’s skin, and cause an endogenic infection. Another source of potential pathogens are microbes which colonise the applied vascular and urinary catheters, drainage tubes, respiration aids as well as other therapeutic and diagnostic equipment (Trautner et al., 2004; Pascual, 2002). The most common etiologic factors that bring about infections of operated tissue are Staphylococcus aureus, Escherica coli, Pseudomonas spp., Klebsiella spp., Enterobacter spp., Bacteroides spp., Candida albicans. In orthopedic patients, due to a broad use of biomaterials, 55% of infection is caused by Staphylococcus epidermidis, and 10–20% by Staphylococcus aureus (Arciole et al., 2005; Husain et al., 1993; Huebner et al., 1990). In fact, S. aureus is an important cause of infection associated with use of metal implants, bone and joint prostheses and cause a soft tissue infections, while S. epiermidis is seen more often in polymer-associated infections (Götz, 2002). Staphylococcus epidermidis growing as biofilm is often very resistant to conventional antibiotics, antiseptics and disinfectants (Brider et al., 2011, Smith et al., 2008). The values of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of antibiotics to bacteria grown in biofilm, may by up to 1000-fold higher compared with bacteria in planktonic form (Høiby et al., 2010). Currently no therapies that target microbial biofilm exist; therefore, new antibiofilm agents, treatments and strategies are needed. The discovery of new antimicrobial agents, active not only against free-swimming microorganisms (planktonic) but also against biofilms, represents an important task (Projan et al., 2002; Karpanen et al., 2008). With this aim, we focused on quaternary ammonium salts
that represent a class of biologically active substances, primary acting as antimicrobial agent (Høiby et al., 2010; Chaieb et al., 2011). Quaternary ammonium compounds are a broad-spectrum bactericides widely used as antiseptics, disinfection and preservation agents (Russel, 2003). The activity of quaternary ammonium compounds, especially the role of long chain against different bacteria, has been previously observed. The positively charged quaternary nitrogen with the polar head groups of acidic phospholipids cause perturbation of cytoplasmic and outer membrane lipid bilayers. Long chain exert antibacterial activity against both Gram-positive and Gram-negative bacteria as well as against some pathogenic species of fungi and protozoa (Ance- lin et al., 2003; Ioannou et al., 2007; McBain et al., 2004).

The aim of this study was to examine the activity of two quaternary ammonium salts against a group of S. epidermidis strains isolated from patients hospitalized in the clinical hospital in Warsaw. These strains growing as plankton, as well as a biofilm structure, were under evaluation. One of the tested compounds was hexa-decylpyridinium bromide (cetylpyridinium bromide), widely known substance with antimicrobial properties, usually used as an antiseptic. The second substance was a newly synthesized derivative: bis-[2-hydroxy-3-(1,7,8,9,10-pentamethyl-3,5-dioxy-4-aza-tricyclo[5.2.1.0(2,6)]dec-8-en-4-yloxy)-propyl]-dimethyl-ammonium chloride (quaternary bis ammonium salt). Generally used quaternary ammonium salts are slightly soluble in water. Newly obtained quaternary bis ammonium salt applied to the tests, has a hygroscopic properties and as very high solubility in the water, so may be used without addition of ethanol to the tested solution.

Synthesis method and basic antimicrobial activity of this agent were previously described (Struga et al., 2008).

Experimental

Materials and Methods

Chemicals. Cetylpyridinium bromide (Sigma) is a known cationic quaternary ammonium compound with antimicrobial activities.

Synthesized quaternary bis ammonium salt was obtained in four steps. First the starting compound (1,2,3,4,5-pentamethylcyclopentadiene) was heated with maleic anhydride. The obtained compound was subjected to the reaction with hydroxylamine hydrochloride in water solution. The product was alkylation with 2-chloromethyl-oxirane and then it was condensed with dimethylamine. Resulting compound was transformed into quaternary ammonium salt by HCl saturated methanol treatment.

Bacterial strains. Twenty three clinical Staphylococcus epidermidis and one reference strain S. epidermidis ATCC 12228 were used in this study. The clinical strains were isolated from following locations: blood (n = 20), catheter (n = 2) and venous catheter (n = 1). S. epidermidis strain ATCC 12228 was used in biofilm assay as a negative control (biofilm non-producing strain). The antibiotic susceptibility of the tested strains was determined by the standard CLSI disk diffusion method (CLSI, 2006a). Twenty one of clinical strains were methicillin-resistant (MRSE). Fifteen strains were resistant to erythromycin, eight – to clindamycin, five – to gentamicin, two – to amikacin, seven – to co-trimoxazole, six – to ciprofloxacin, three – to tetracycline. All strains were susceptible to linezolid.

Microorganisms were derived from the collection of Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

Antibacterial susceptibility testing – MIC method. The minimum concentrations of both tested quaternary ammonium compounds inhibiting growth of S. epidermidis strains (MICs) were determined by reference broth dilution methods using Mueller-Hinton II Broth, according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2006b).

Concentrations of tested compounds in liquid medium ranged from 0.0625 to 256 µg × mL⁻¹, cetylpyridinium bromide was dissolved in 50% ethanol, quaternary bis ammonium salt – in distilled water. The final inoculum of all microorganisms studied was about 10⁴ CFU × mL⁻¹ (colony forming units per mL). MICs were read after 18 h incubation at 35°C. The MIC values determined in susceptibility test on planktonic bacteria were applied also during staphylococcal biofilm susceptibility determination.

S. epidermidis biofilm susceptibility testing – MTT method. All strains were kept frozen at −70°C in BHI broth supplemented with 10% glycerol, until use. The strains were first grown from a frozen stock on BHI agar plates by incubation during 24 h at 37°C. Bacterial colonies were harvested, and suspended in 0.85% NaCl to prepare cells suspensions for biofilm production. The density of all cell suspensions was adjusted to the value of 3.2 units in McFarland scale, which was equal to 10⁴ CFU × mL⁻¹. Each inoculum was diluted 1 to 10 with fresh BHI broth. Individual wells of polystyrene, sterile 96-well tissue culture plates (Kartell S.p.A., Medlab, Italy), were filled up with 200 µL of diluted cultures and incubated for 24 h at 37°C. Additionally 200 µL of BHI medium was added to some wells, as control of sterility and non-specific media binding.

After incubation planktonic cells were removed and bacterial biofilm layer was treated with quaternary bis ammonium salt at concentrations ranging from 2.0 to 128.0 µg × mL⁻¹. For comparative and quality control purposes, other wells with biofilm were treated with quaternary ammonium derivative – cetylpyridinium
bromidum – at concentrations from 0.125 to 8.0 µg × mL⁻¹. The plates were incubated with these compounds for 18 h at 37°C. Following incubation period, the analyzed compounds were removed from the microplate wells and they were washed with sterile phosphate buffer saline (PBS). Alive bacterial cells were stained with 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT; 0.5% in PBS) for 2 hours at 37°C. In parallel, appropriate bacterial biofilm not treated with these two compounds was used as a control. After 2h of staining, solution was removed and biofilm was solubilized by 150 µl DMSO with 25 µl glycine buffer (0.1 M × L⁻¹, pH 10.2) treatment. Solution absorbance was measured at 554 nm wavelength using a PowerWave XS (BioTek) spectrophotometer.

Comparing the average results of absorbance \(A_{554}\) of the control biofilm with the biofilm treated with tested compounds, the percentage of killing efficiency for each concentration and for each strain, was calculated by the following formula:

\[
\text{Percentage of killing efficiency} = \frac{A_{554, \text{control biofilm}} - A_{554, \text{treated biofilm}}}{A_{554, \text{control biofilm}}} \times 100%
\]

**Results**

The MIC values for the cetylpyridinum bromide for planktonic cells ranged from 0.125 µg × mL⁻¹ to 8.0 µg × mL⁻¹. For the almost half of strains (11/24–46%) the MIC value was 2.0 µg × mL⁻¹. For the references strain *S. epidermidis* (ATCC 12228) this value was 0.125 µg × mL⁻¹. Only for the one tested strain MIC value was 8.0 µg × mL⁻¹. For the quaternary bis ammonium salt MIC values for the planktonic cells ranged from 16.0 µg × mL⁻¹ (only one – references strain) to 128.0 µg × mL⁻¹ (14/24–58.3%).

The average value of absorbance was calculated for each strain, after treatment of tested compounds.

Analysis of results for testing strains during experiment with cetylpyridinum bromide, allowed to classify these strains as low, intermediate and high biofilm producers, depending on absorbance value. The absorbance levels \(A_{554}\) were assumed subjectively by authors in order to classify the analysed strains into 3 groups of different biofilm production level. First group: high biofilm producers – absorbance values above 1.5; second group: intermediate biofilm producers – absorbance 1.0–1.5, and third group: low biofilm producers – absorbance below 1.0. Only few (3/24) strains were classified as high biofilm producers, whereas 13 of tested strains displayed low biofilm production phenotype. The second group of eight strains, including *S. epidermidis* ATCC 12228, previously described as non-biofilm producer [23]. Average absorbance value for this strain was 1.172.

The percentage of killing efficiency was calculated for each strain. In 0.125 µg × mL⁻¹ concentration of cetylpyridinum bromide, the percentage of killing efficiency was 43.6% for the first group of strains, 62.7% for second group, and 44.7% for third group. In the highest concentration 8.0 µg × mL⁻¹ this efficiency was 48.3%, 64.1% and 47.0% respectively. Medium values of killing efficiency ranged from 26.6% to 64.1% for all tested concentrations in three groups of strains. The most sensitive for cetylpyridinum bromide were strains from intermediate biofilm producers group (48.6–64.1% of killing efficiency). The most resistant were bacterial strains from third group – low biofilm producers (26.6 to 47.0% of killing efficiency).
Fig. 2. Values of biofilm absorbance of *S. epidermidis* strains groups: low biofilm producers ($A_{554} < 1.0$), medium biofilm producers ($1.0 < A_{554} < 1.5$), high biofilm producers ($A_{554} > 1.5$), strain 340/08 and references strain ATCC 12228, after treatment with quaternary bis ammonium salt.

Fig. 3. Values of biofilm absorbance of *S. epidermidis* strain 340/08, after treatment with cetylpyridinium bromide (A) and quaternary bis ammonium salt (B).

strains from the second and third group the most effective concentration was 8.0 µg × mL⁻¹, while for the first group the most effective was 0.25 µg × mL⁻¹. For the all tested *S. epidermidis* strains the most effective was the highest used concentration – 8.0 µg × mL⁻¹ (average about 53% of killing efficiency) (Fig. 1).
These results showed that cetylpyridinium bromide is activity towards S. epidermidis biofilm in vitro, regardless of the concentration. None of the concentrations used caused total eradication of staphylococcal biofilm, but the clear limiting effect on the growth was observed.

During the experiment with quaternary bis ammonium salt the analyzed strains were again classified to three biofilm producers groups, as it was described above. In this part of research some strains were classified to different group as it was previously. To first group – high biofilm producers 9 strains were classified, to second – intermediate biofilm producers – 9 strains, and to third – low biofilm producers – 6 strains. The references strain in this part displayed low biofilm producers phenotype (average absorbance value 0.943).

For each strain the percentage of killing efficiency for quaternary bis ammonium salt was also calculated. Average killing efficiency ranged from 59.7 to 88.4% for tested concentrations (from 2.0 to 128.0 µg × mL\(^{-1}\)) in all groups. The intermediate biofilm producers (74.3 to 88.4% of killing efficiency) were the most sensitive to this compound, while the low biofilm producer strains were the most resistant (59.7 to 80.3%). For all tested strains the most effective concentration was 128.0 µg × mL\(^{-1}\) (Fig. 2).

Among most of tested strains increased concentration of quaternary bis ammonium salt, caused higher anti-biofilm efficiency. The most resistant strain for cetylpyridinium bromide and quaternary bis ammonium salt treatment was strain 340/08 (Fig. 3). Only the highest concentrations of quaternary bis ammonium salt slight decrease of absorbance values, and thus decreased of quantity of viable bacterial cells. For cetylpyridinium bromide was not observed such a clear effect on bacterial biofilm.

**Discussion**

In this study two quaternary ammonium salts were examined against planktonic cells and biofilm of clinical Staphylococcus epidermidis strains. Cetylpyridinium bromide is widely used as an antiseptic compound. The second analyzed agent – newly synthesized derivative called quaternary bis ammonium salt.

There was no previous reports about activity of these two compounds against S. epidermidis biofilm and planktonic cells. Most of publications are focused on other widely used, chemical antiseptics – chlorhexidine, triclosan, alcohols, benzalkonium chloride (Karpanen et al., 2008; Chaieb et al., 2011; Russel, 2003; Ioannou et al., 2007). During this study cetylpyridinium bromide proved to be more effective compound, compared with a newly synthesized quaternary bis ammonium salt. Activity of this derivative towards planktonic bacteria was comparable with commonly used triclosan and chlorhexidine. However, MICs values for quaternary bis ammonium salt showed, that it can also act a very effective antimicrobial agent. It has been shown that cetylpyridinium chloride for example used in mouth rinsing preparations, interacts strongly with Streptococcus mutans biofilm. This compound diffuses slowly in biofilm structures and appears to bioaccumulate forming a structure that practically cannot be washed out from biofilm (Marcotte et al., 2004).

Biofilm development is a dynamic and variable process, dependent on many different external factors, and it is nearly practically impossible to obtain in vitro in a reproducible manner biofilm level from the same strain. S. epidermidis biofilms were more resistant to both of used compounds compared to planktonic form of the same strains. None of the tested compounds was able to completely eradicate the biofilms structures attached to surface of polystyrene plate wells.

Smith and Hunter (Smith et al., 2008) conducted a study on the effects of 1% benzalkonium chloride, 4% chlorhexidine digluconate and 1% triclosan against biofilm formed by clinical multidrug resistant isolates of S. aureus and P. aeruginosa. The same concentrations of tested compounds were chosen as used for disinfection in hospitals. For these compounds the killing efficiency was: for S. aureus – from 89% to 100%, for P. aeruginosa – about 20% (Smith et al., 2008). The results from this study show, that bacterial cells in biofilm structure are very resistant, despite that concentrations of tested compounds were higher than used in our study.

Investigation on the bactericidal activity of the tested compounds: cetylpyridinium bromide and a newly synthesized derivative: quaternary bis ammonium salt, against biofilms of S. epidermidis strains, confirmed the literatures data (Smith et al., 2008; Chaieb et al., 2011). Bacteria present in the biofilm exhibit significantly greater resistance for antibacterial agents, in comparison with planktonic forms of the same strains.

None of these compounds caused total eradication of staphylococcal biofilm in all tested concentrations.

**Literature**


Chaieb K., T. Zamantar, Y. Souiden, K. Mahdouani and A. Bakhouf. 2011. XTT assay for evaluating the effect of alcohols,
**Introduction**

Filamentous fungi are renounced for their ability to produce bioactive secondary metabolites that can affect organisms inhabiting the same ecological niche. This is also true for the genus *Fusarium* which has been reported to produce ecotoxins, phytotoxins, growth inhibitors and antibiotics (Chełkowski, 1989; Kwaśna and Chełkowski, 1991). An especially interesting group of *Fusarium* metabolites are the mycelium pigments aurofusarin, bikaverin, fusarubins and intermediates in their corresponding biosynthetic pathways. Several of these metabolites have been shown to have antibiotic properties and may, in future, be put to practical use. However the physiological basis for the synthesis of these compounds, the role of the pigments for the producing organism, the genetic background for the process as well as the effects on living organisms other than bacteria should be comprehensively researched. However, the majority of *Fusarium* sp. are plant pathogens that pose a huge problem in agricultural production, as infections of cereals crops by these fungi results in reduced harvest yields and the accumulation of toxic secondary metabolites dangerous for humans and animals (Chelkowski, 1989; Kwaśna and Chelkowski, 1991; Parry et al., 1995; Schaafsma et al., 2001; Bottalico and Perrone, 2002; Logrieco et al., 2002; Pirgozliev et al., 2003; Snijders, 2004; Desjardins, 2006; Rep and Kistler, 2010). Members of the *Fusarium* genus are typically identified by their red or brown mycelium coloration and banana shaped conidia (Kwaśna et al., 1991; Leslie et al., 2006). The red coloration is due to the production of either the monomeric polyketide bikaverin (Cornforth et al., 1971; Kjaer et al., 1971; Linnemannstöns et al., 2002), the monomeric polyketide fusarubin (Pari sot et al., 1990) or the homodimeric polyketide auro fusarin (Medents e v and Akimenko, 1998; Medents ev et al., 2005). Aurofusarin is among others produced by species belonging to the *F. graminearum* species complex. The biosynthesis is dependent on a 25 kb gene cluster, consisting of ten genes (PKS12, aurR1, aurO, aurT, aurR2, aurZ, aurJ, aurF, GIP1 and aurS). Targeted gene replacement studies of these genes have facilitated the development of a theory for the biosynthetic pathway of aurofusarin (Medents ev et al., 2005; Malz et al., 2005; Frandsen et al., 2011). The pathway includes at least six enzyme catalyzed steps and four stable intermediates have been identified and structurally...
characterized; YWA1, nor-rubrofusarin, rubrofusarin and fuscofusarin. The presence of signal peptides (export signals) in three of the involved enzymes suggest that the last steps of the biosynthetic pathway is catalyzed extracellularly. However the accumulation of rubrofusarin in several of the constructed strains, ΔaurF, ΔaurS and ΔGIP1, has hampered mapping of the last steps that converts rubrofusarin into aurofusarin (Frandsen et al., 2006).

Apart from F. graminearum the most common pathogenic Fusarium sp. found in moderate climate cereals is F. culmorum. The F. culmorum species is characterized by a wider feeding spectrum and is deemed to be a pathogen of many vascular plants and edible mushrooms. It may also occur within potato stores and is commonly found in soil in the form of saprophytes. F. culmorum, similar to F. graminearum, is considered to be highly toxic and its mycelia is intensively coloured (the reverse side of mycelia in cultures is carmine red) (Kwasina et al., 1991; Kang and Buchenauer, 2002; Jackowiak et al., 2005). The biosynthetic basis for aurofusarin formation in F. culmorum is identical to what has been described in F. graminearum (Malz et al., 2005).

Nanosilver is characterized by its strong antibacterial activity which is owed to its large surface area to weight ratio and its effects therefore depends on the size of nanoparticles (Lee et al., 2005; Morones et al., 2005; Panacek et al., 2006; Kim et al., 2007; Pal et al., 2007; Shrivastava et al., 2007; Lewinski et al., 2008; Martinez-Castanon et al., 2008; Tien et al., 2008; Kim et al., 2012). The antibiotic effects of nanosilver has been linked to its interaction with thiol groups in proteins, affecting the replication of DNA, and its ability to uncouple the electron transport chain from oxidative phosphorylation in the mitochondrial membrane and by collapsing the proton-motive force across the cytoplasmic membrane (Holt and Bard, 2005; Marini et al., 2007). Toxic effects have also been reported for eukaryotic cells and literature includes data for both human cell lines (T, neuron, liver, skin, lung epithelial cells, and macrophages) and fungi cells. For human or animal cells it is reported that silver nanoparticles exerts cytotoxicity through the generation of reactive oxygen species resulting in induction of programmed cell death (apoptosis). Apoptosis eliminates damaged or unwanted cells via nuclear condensation, membrane blebbing, and DNA fragmentation in the process of development or in response to infection or DNA damage. Nanosilver has also been shown to deplete the antioxidant glutathione pool, cause mitochondrial dysfunction, and induce the release of lactate dehydrogenase (Hussain et al., 2005; Keuk-Jun et al., 2008; Asharani et al., 2009; Foldbjerg et al., 2009; Kim et al., 2009; Miura and Shinohara, 2009; Yen et al., 2009; Eom and Choi, 2010; Samberg et al., 2010; Yang et al., 2010). Nevertheless, ongoing projects aims to investigate a variety of clinical applications of nanosilver such as delivery vehicle for genes and drugs, biosensors, implantable materials, and bone prostheses, and as drugs against skin infecting fungi (dermophytes) (Ahmed et al., 2010; Faunce and Watal, 2010). Nanosilver’s current applications are mainly focused on its antibacterial properties and it is used in personal care products, household appliances, antiseptic materials (Margaret et al., 2006; Rujitanaroja et al., 2008; Dastjerdi et al., 2009), water purification (Pradeep and Anshup, 2009) and food packaging (Tankhiwale and Bajpai, 2009).

The conducted research aimed for an initial cognitive evaluation of the in vitro effect of nanosilver on a Polish F. culmorum isolate as well as an analysis of metabolites in fungal cultures subjected to contact with silver nanoparticles. Preliminary tests carried out may indicate whether the well-known nano-antibacterial activity may be useful as inductor of bioproduct synthesis like fungal metabolites.

**Experimental**

**Materials and Methods**

**Used nanosilver.** Silver nanoparticles have been obtained by the use of a method based on high voltage electric arcs between silver electrodes. A detailed description of the method and the apparatus used to produce the nanoparticles has been described by Kasprowicz et al. (2010). The silver nanoparticles size ranged between 15 and 100 nm. Nanomaterial used is shown in Fig. 1.

**Fungal strains and culture conditions.** F. culmorum strain was isolated from red cabbage (Brassica oleracea subsp. capitata f. rubra) in 1997 and purchased from the collection of the Plant Protection Institute in

![Fig. 1. TEM image of silver nanoparticles.](image-url)
Poznań (Poland). The fungus was cultured on solid SNA medium in natural photoperiod conditions until intensively sporulating mycelium developed. Synthetic nutrient agar (SNA) medium – a base for obtaining abundant sporulation of the fungus Fusarium sp. composed of: 1 g KH₂PO₄; 1 g KNO₃; 0.5 g MgSO₄·7 H₂O; 0.5 g KCl; 0.2 g glucose; 0.2 g saccharose; 0.6 ml NaOH (1 M) and 13.2 g agar in 11 of distilled water. A concentrated sterile water suspension of the fungus spores (about 5·10⁶ pcs conidia · ml⁻¹) was prepared. The number of spores was estimated using a Bürker chamber. Smears of fresh spore suspension were placed on solid culture media in sterile 90 mm diameter Petri dishes using sterile swabs. Immediately after smearing, 4 sterile 10 mm diameter tissue paper discs were placed in the Petri dish saturated with silver nanoparticles of 7, 35 and 70 ppm concentration. The control group consisted of dishes containing spore smears with sterile distilled water saturated discs placed on top. The resulting cultures were incubated at 21°C in an incubation chamber for 14 days and a natural photophase. Experiments was carried out on standardized solid PDA medium (Biocorp company, Warszawa, Poland) comprising of potato extract 4 g · l⁻¹, glucose 20 g · l⁻¹ and agar 15 g · l⁻¹. Strained medium was also used, containing half of the values per litre of the above mentioned ingredients. The plate based experiment was conducted with seven replicates. Following incubation the pigmentation on the reverse side of the mycelium was established using an Agfa Duoscan T 1200 (Agfa – Gevaert N.V., Belgium) scanner and computer analysis. The pictures were processed using the UTHSCA Image Tool program v. 3.0 (The University of Texas Health Science Center at San Antonio, Texas, USA). Picture processing comprised of separating the RGB components and selecting the green hue which was exhibited the strongest contrast between the background and the areas where the dye occurred, using median filters, opening and closing, histogram equalization, limiting grey hues, single step binarization, labelling objects and measuring the number of pixels in a given object (Fig. 2).

The F-Snedecor single factor analysis of variance test for the area of occurrence of dye in experiments was used to a statistical analysis.

**Analysis of metabolites.** Five mm agar plugs were cut out of the agar plates and metabolites were extracted and prepared for HPLC-DAD analysis as described in Smedsgaard (1997). The samples were analysed on a Hewlett-Packard Model 1100 HPLC equipped with a diode array detector (200–500 nm) and a GROM-SIL 120 ODS-5ST, 3 mm, 60 x 4.6 mm column (Grom Analytik +HPLC GmbH). Ten microliter samples were introduced onto the column with an isocratic flow of 20% acetonitril and then eluted on a linear gradient to 80% acetonitrile over an 8 minutes period. The eluents were adjusted to 0.1% O-phosphoric acid. Aurofusarin, rubrofusarin and nor-rubrofusarin were identified by comparing with published UV-spectra (Frandsen et al., 2006; Shoji et al., 1967; Eisaku et al., 1968) and metabolites extracted from F. graminearum PH-1 (wild type), ΔaurF and ΔaurJ mutants published in Frandsen...
et al. (2006; 2011). The concentration of the extracted metabolites was normalized to the fungal biomass, by measuring the ergosterol concentration in the extracts. The ergosterol concentration was determined by injecting 10 µl of sample on the same column as described above, but elute with an isocratic flow of 100% acetonitril + 0.1% O-phosphoric acid, as described in Frandsen et al. (2006). Ergosterol was quantified by comparing to 100, 200, 500, 750 and 1000 µg ∙ ml⁻¹ ergosterol standards (Invitrogen). The incubations, extractions and LC-DAD analysis were performed in triplicates.

Results and Discussion

The disk-diffusion method is a research test commonly employed in order to establish the sensitivity of bacteria or fungi to a given compound. This method has previously been used in other studies to evaluate the effect of nanosilver on fungal growth. Petica et al. (2008) as well as Gajbhiye et al. (2009) have both reported a clear growth inhibition of several fungal species. We have previously reported (Kasprowicz et al., 2010) the effects of nanosilver colloids on concentrated water solutions of the same F. culmorum isolate as used in the current study, where we observed an intensive inhibition of mycelia surface growth cultured from spores after contact with nanosilver. The vegetative mycelia obtained from spores, that had been in contact with nanosilver was also characterised by a significantly more intensive sporulating as compared to the control culture. The next modified characteristic of the fungus was the germination of spores. We concluded a decrease in the number of germinating spores as well as the length of germ tube for the nanosilver group as compared to the control group. The research was conducted for low concentrations levels of nanosilver (0.12–2.5 ppm). Panacek et al. (2006) in testing fungistatic effects of nanosilver colloids on Candida albicans observed an inhibition of fungal growth and development for very low nanosilver colloid concentrations, with no cytotoxicity for human fibroblasts, which provides a basis for further research on the practical uses of nanosilver in treating human mycosis. The results obtained to date permit one to conclude that nanosilver stem the life functions of fungi.

In the conducted experiment, a significant stimulation of pigment biosynthesis in F. culmorum was observed when the fungus was exposed to nanosilver in concentrations of 35 and 70 ppm. The heavily pigmented surface of the reverse side of the mycelium grown on full PDA media with the above mentioned concentrations was three times as large as that observed in the control group. At the lower concentration, 7 ppm nanosilver, the mycelia pigmentation was comparable with that of the control group. The results obtained for the pigmented area of mycelia cultures on a standard PDA medium is shown in Fig. 3. It was concluded that together with the increase in nanosilver concentrations in saturated disks placed over the mycelia, the area of pigmentation on the reverse side of cultures increased significantly, which is attributed to a modification of the physiological processes in the fungus in the presence of nanosilver.

Table I depicts a statistical analysis of variance for the obtained results. The lack of statistically significant differences in the pigmentation of cultures as compared to the control group was only obtained for the lowest nanosilver concentration levels of 7 ppm. The higher concentration levels, which are 35 and 70 ppm, of nanosilver significantly, modified the culture pigmentation as compared to the control group.

In cultures on strained PDA media, the control cultures displayed the smallest area of discolouration. The use of nanosilver stimulated the evaluated fungal strain to biosynthesise pigments at significantly elevated levels as compared to that of the control group, an effect that was dose dependent. The results obtained are illustrated in Figure 4. Statistical analysis of variance (Table II) indicated that only for nanosilver con-
Nanosilver influence on pigments production by *F. culmorum*

Concentration levels of 35 and 70 ppm were the obtained pigmented areas similar.

The effects of nanosilver on mycelium pigment production in *F. culmorum* was analysed by LC-DAD. The addition of 70 ppm nanosilver on a paper disk test changed the mycelium colour from yellow to red. Nanosilver stimulates the conversion of rubrofusarin into aurofusarin, but whether this conversion was due to a direct catalytic effect of nanosilver or due to stimulation of the natural enzyme system is unknown. However, the addition of nanosilver also resulted in the accumulation of a novel pigment (rt = 3.504 min) with a UV/VIS spectrum very similar to that of aurofusarin (Fig. 6 C and D). This compound has not been observed previously in the natural system, pointing towards both a biotic and abiotic effect of nanosilver. The novel compound eluted earlier than aurofusarin under the used reverse phase chromatography conditions suggesting that it is more hydrophilic than aurofusarin. This combined with the similarities in the UV/VIS spectrum suggest that the novel compound is a dimer of one of the previously described intermediates in the biosynthetic pathway, such as YWA1, nor-rubrofusarin, rubrofusarin or combinations of these. Definitive identification of this compound will rely on NMR and HR-MS studies. However there is no doubt that the unknown compound is structurally related to aurofusarin (dimeric), based on the high degree of similarity to the aurofusarin UV-spectrum. The UV spectrum is primarily the result of the extensive conjugated bond system within the molecule.

The toxic effects of nanosilver have been suggested to be mediated by their production of reactive oxygen species (ROS), such as superoxide radicals (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH$^*$) (Gajbhiye et al., 2009; Ivask et al., 2010). An increased concentration of extracellular radicals might explain the formation of the novel aurofusarin related metabolites in the current experiments. Dimerization and polymerization of fungal pigments has been linked to radical mediated activation of monomers, known as oxidative coupling (Gill et al., 1990), this is also the case for the aurofusarin biosynthetic pathways where the monooxygenases (AurF) or laccases (GIP1) are responsible for catalyzing the dimerization of two monomers. The biological function of polyaromatic pigments for fungi is in part to protect from damage caused by VIS/UV-radiation, by absorbing light, and secondly to act as an antioxidant defence mechanism that quenches radicals, formed by both abiotic and biological mechanisms. Future experiments will be directed towards understanding whether the effects of nanosilver is due to ROS that affects the biosynthetic apparatus, or whether the effect is more directly based on the formation of radical forms of the aurofusarin intermediate, which might facilitate premature dimerization of intermediates to form non-natural intermediates.

**Conclusion.** Nanosilver modifies the metabolism of the analyzed *F. culmorum* strain. Coming into contact with nanosilver colloids induces more intensive mycelia...
pigmentation correlated with nanosilver concentration levels. The performed analysis of metabolites indicates that under the influence of nanosilver fungi biosynthesize aurofusarin more intensively and the conversion of rubrofusarin to aurofusarin is intensified as compared to the control culture. Under the influence of nanosilver F. culmorum intensively biosynthesises an un-identified dye with a UV/VIS spectrum that is similar to that of aurofusarin, though not produced by fungi in standard cultures. Achieved preliminary results for Polish strain of Fusarium fungus indicate that in addition to its antibacterial and fungistatic nanosilver can be used for induction of biosynthesis specific dyes of fungi. However, this requires further detailed study in relation to other species and strains of Fusarium fungi, as well as recognition of the direction of changes in biosynthesis.

Acknowledgments
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Literature


Possibilities in Identification of Genomic Species of *Burkholderia cepacia* Complex by PCR and RFLP

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**Abstract**

The strains belonging to *Burkholderia cepacia* complex are important opportunistic pathogens in immunocompromised patients and cause serious diseases. It is possible to obtain isolates from soil, water, plants and human samples. Taxonomy of this group is difficult. *Burkholderia cepacia* complex consists of seventeen genomic species and the genetic scheme is based on recA gene. Commonly, first five genomovars occur in humans, mostly genomovars II and III, subdivision IIIA. Within this study we tested identification of first five genomovars by PCR with following melting analysis and RFLP. The experiments were targeted on eubacterial 16S rDNA and specific gene recA, which allowed identification of all five genomovars. RecA gene appeared as more suitable than 16S rDNA, which enabled direct identification of only genomovars II and V; genomovars I, III and IV were similar within 16S rDNA sequence.

**Key words:** recA, 16S rDNA, HRMA

**Introduction**

Mapping and dissemination of infection agents are an important topic in public health and epidemiology. Molecular methods have contributed to recent advances in the tracking of the nosocomial and environmental spread of pathogenic bacteria. These methods enable genetic identification of microbes at the level of subspecific strains (Belkum *et al.*, 2002).

*Burkholderia cepacia* complex (Bcc) is a very diverse group of bacteria, whose members are opportunistic human pathogens that can cause infections in immunocompromised patient, for example with cystic fibrosis and in other people with immune deficiency (Govan *et al.*, 1996). Species from the Bcc can be transmitted between patients and are frequently resistant to wide range antibiotics. In addition, infection with these strains can cause a “cepacia syndrome” or a necrotizing pneumonia with bacteremia which leads to an acute and frequently fatal clinical decline (Frangolias *et al.*, 1999; Isles *et al.*, 1984). Distinction of genomic species in the Bcc by routine clinical microbiology methods is difficult. There are available phenotypic tests to identify genomovar II (species *B. multivorans*) and III (species *B. cenocepacia*) as infections caused by genomovar II can evolve into fatal “cepacia syndrome”. The two above mentioned genomovars are more virulent than other belonging to complex. Identification of genomovars can be useful for treatment purposes (Zahariadis *et al.*, 2003; Nash *et al.*, 2011).

Aim of our study was to verify an applicability of the previously published systems for identification of genomovars belonging to the Bcc (Whitby *et al.*, 2000; Mahenthiralingam *et al.*, 2000 and Dřevínek *et al.*, 2002). These systems use agarose gel electrophoresis as post-PCR analysis, however, we verified possibility of using melting analysis, which is closed system, so there is no possible cross contamination, reduced sample handling and the method is less time-consuming.

**Experimental**

**Materials and Methods**

**Bacterial isolates.** 145 clinical isolates from collection of Department of Microbiology, Faculty of Medicine and Dentistry, Palacký University Olomouc and Faculty Hospital Olomouc and 5 reference strains from
The Belgium Coordinated Collections of Microorganism/Laboratorium Microbiologie Ghent (BCCM/LMG) LMG 1222, LMG 13010, LMG 16656, LMG 14294, LMG 10929 were used in this study. Strains were cultivated overnight on blood agar for DNA extraction.

**DNA extraction.** Chromosomal DNA was extracted from all strains by using heat extraction. First, colony was resuspended in 50 μl of deionized water, incubated at 94°C for 10 min, centrifuged by 13 000 × g for 4 min and then 25 μl of supernatant was transferred into new tube. Extracted DNA was used directly as template for PCR or kept at –20°C until needed.

**PCR analysis.** Each 20 μl reaction contained 14.24 μl deionized water, 2 μl buffer complete (final concentration of MgCl₂ was 1.5 mM), 1 μl LCGreen Plus, 0.1 μl of primer (final concentration 0.5 μM, primer pair to PCR system choose according Table I), 0.16 μl dNTPs (25 mM stock, final concentration 0.2 mM), 0.4 Taq-polymerase (2 U/reaction) and 2 μl DNA template. For real-time PCR amplification with following High-resolution melting analysis (HRMA) was performed using Rotor-Gene 6000 (Qiagen). Thermal cycling parameters for PCR system A and C included a pre-denaturation at 94°C for 7 min followed by 40 cycles of 30 sec at 94°C (denaturation), 45 sec at 62°C (annealing) and 60 sec at 72°C (extension) and final extension 7 min at 72°C and melting in range from 55°C to 95°C. Fluorescence data were acquired at 0.03°C increments.

Rotor-Gene software (version 2.0.2.4) enabled visualisation of HRM data. The negative derivative of fluorescence over temperature (df/dt) curve displays melting temperature (Tm), the normalized curve represented the decreasing fluorescence versus increasing temperature (Wittwer et al., 2003). For distinguish of strains we used derivative curves with melting temperature (Tm).

Thermal cycling parameters for PCR system B included a pre-denaturation at 94°C for 7 min followed by 30 cycles of 45 sec at 94°C (denaturation), 45 sec at 66°C (annealing) and 120 sec at 72°C (extension) and final extension 7 min at 72°C.

Results from PCR system B were evaluated on the basis of negativity or positivity, according algorithm to identify genomovars of the Bcc, published earlier in Whitby et al. (2000).

For restriction fragment length polymorphism (RFLP) analysis, 10 μl of PCR product amplified by PCR

<table>
<thead>
<tr>
<th>PCR system</th>
<th>Specificity</th>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Target</th>
<th>Reference</th>
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<tr>
<td>A Genomovar I (B. cepacia)</td>
<td>BCRG11</td>
<td>CAGGTCGCTCTCCACGGGT</td>
<td>recA</td>
<td>Mahenthiralingam et al., 2000; Dřevínek et al., 2002</td>
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<td>Whitby et al., 2000</td>
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</table>
Identification of genomic species of *Burkholderia cepacia* complex

System C was combined with deionized water (17 μl), the appropriate restriction enzyme buffer (2 μl) and endonuclease *HaeIII* (1 μl) and incubated at 37°C for 7 min. RFLP products were analysed by agarose gel electrophoresis, with agarose concentration 2%. Molecular size marker of the appropriate size range was included on all gels (100 bp DNA ladder or 50 bp DNA ladder).

**Results**

We tested the utility of PCR and RFLP to distinguish first five genomovars of Bcc. These methods were tested on 145 clinical isolates and five reference strains from BCCM/LMG. Only one clinical isolate belonged to genomovar I, 109 clinical isolates to genomovar II and 35 to genomovar III, subdivision IIIA.

Genomovar specific amplification (PCR system A), Bcc specific PCR, which was aimed on recA gene, provided higher resolution capability. This method enables to distinguish all five tested genomovars and within genomovar III it was possible differentiate two subdivisions IIIA and IIIB. The identification by the PCR system A was based on the presence of the specific peak (Fig. 1). Results from melting analysis were supported with results from agarose gel electrophoresis, for verify usability of melting analysis for determination positive or negative reaction.

The system published by Whitby *et al.* (2000) (PCR system B) targeted 16S/23S rDNA within two PCR with primer pairs G1-G2 and SPR3-G1 or SPR4-G1. Primer pairs G1-G2 enabled to distinguish genomovars I, III and IV from genomovars II and V. Second PCR with primer pair SPR3-G1 or SPR4-G1 allowed identification of genomovars I/III, I/IV, II and V. It was possible to distinguish genomovars II and V according the PCR system B.

PCR system C with following RFLP analysis with *HaeIII* of PCR-amplicons of 16S rDNA revealed sequence polymorphism capable of identifying genomovars II and V but was insufficient to discriminate genomovars I, III and IV (Fig. 2). RFLP analysis with *HaeIII* of PCR-amplicons of recA gene was enough sufficient nucleotide sequence variation to distinction of all tested genomovars I, II, III, IV and V (Fig. 3). Isolates of genomovar III included two the subdivision IIIA and IIIB after cleavage recA by *HaeIII*.

At first we demonstrated that the melting analysis can replace agarose gel electrophoresis as post-PCR analysis. Analysis of recA gene by the PCR system A with following melting analysis or RFLP achieved the same discrimination power, and analysis of 16S rDNA by the PCR system B with following melting analysis or RFLP gave the same results. The differentiation of genomovar II (species *B. multivorans*) was possible by using the PCR system A, B or RFLP aiming 16S rDNA and recA genes. Genomovar III (species *B. cenocepacia*) was directly identified only by genomovar specific PCR (PCR system A) or RFLP with *HaeIII* aimed at recA gene. Finally, we proved that the analysis of specific gene is more suitable for identification and distinction of Bcc than eubacterial gene, which we can find by all bacterial species.
Discussion

Several studies have indicated problems with right identification of Bcc by phenotypic methods. The molecular methods are more reliable in this field (Henry et al., 1997; McMenamin et al., 2000). Within the genetic identification we aim for molecular marker recA gene and 16S rDNA, which is very extensive for high degree of conservation and included variable regions (Liu et al., 2012).

Our results of PCR with following melting analysis or RFLP analysis were same as in studies Whitby et al., 2000; Mahenthiralingam et al., 2000a; McDowell et al., 2001 and Mahenthiralingam et al., 2002. Studies Mahenthiralingam et al. (2000b) and Dřevínek et al. (2002) describing the development of genomovar specific PCR. We tested their conclusions in practice and we compared it with PCR and RFLP aimed at eubacterial 16S rDNA. Genomovar specific PCR and RFLP focused on recA gene had better discriminating power.

We had not enough isolates of genomovar IIIB and IV (they are not easy accessible) so we cannot surely conclude that according results from melting analysis that the distinction of curves appertain to genomovar IIIB and IV is significant. We recommend verifying the results of identification of genomovars by agarose gel electrophoresis, size of PCR product is different, for IIIB is 781 bp and for IV is 647 bp. Whitby et al. (2000) developed PCR reactions focused on 16S rDNA and 23S rDNA for identification of Bcc. PCR was scored based on positive and negative reactions, described method enabled to distinguish genomovars II and V, which had differences in sequence of 16S rDNA and it was possible to suggest primers for dissimilar regions. Sequences of genomovar I was similar to genomovar II and IV.

We focused our experiments only on first five genomovars, which occur with the highest frequency. Their identification and discrimination would be beneficial as it includes two genetic types also that cause most complications in infected (occurrence of antibiotic resistance, cepacia syndrome). These are genomovar II and genomovar III and they are two the most commonly isolated genomovars too. These two genomovars are significant for epidemiology (Mahenthiralingam et al., 2002 and Jones et al., 2003).

Acknowledgments

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Literature


Introduction

Complicated urinary tract infections (UTIs) are associated with structural or functional abnormalities in the urinary tract such as a catheterization, calculi, immunosupression and others (Jacobsen, 2008). Probability of developing the infection is similar in women and men and in all age groups (Pallet and Hand, 2010). Devices, e.g. indwelling catheters, which are inserted in the urinary tract facilitate microbial colonization. Bacteria attach to outer and inner surfaces of a catheter and form biofilm, which is responsible for 65–85% catheter associated UTIs. After a few days of catheterization multi-species biofilm, consisting of several organisms, is often isolated (Muzzi-Bjornson and Macera, 2011). Species like: Enterococcus faecalis, Staphylococcus epidermidis, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae are most frequently detected on the catheter surface (Niveditha et al., 2012). The structure of multi-species biofilm is different from biofilm formed by one species of bacteria. The interactions between microorganisms which form this structure change biofilm physiology and function, facilitating the horizontal gene transfer. Consequently, in multi – species biofilms resistance to antimicrobial agents and host defense is observed more frequently (Yang et al., 2011). Biofilm age, its density and the extent of the surface which it covers are the main factors affecting its susceptibility to antimicrobials. It is possible that microorganisms in biofilm use a number of mechanisms responsible for their resistance. The first one is slow or incomplete penetration of antimicrobial agents into biofilm. A significant role in this process is played by a sticky matrix which covers bacterial cells and prevents the diffusion of drugs to the target by binding them or influencing the rate of
molecules transport to the biofilm interior. An explanation for high biofilm resistance could be the reduction of the metabolic activity of sessile bacterial cells. A significant role in biofilm resistance to antibiotics is played by persisters: a spore-like cells, dormant and incapable of growth (Hoiby et al., 2010, Lazar and Chifiriuc, 2010). The main mechanism is still undetermined, recognition of this process will help to develop new methods of preventing biofilm formation and matriculation processes. Numerous mechanisms of biofilm resistance cause that concentrations of antibacterial agents needed for biofilm eradication are 1000–1500 times higher than concentrations needed to eradicate planktonic bacteria of the same species (Chen and Wen, 2011). Urea-splitting bacteria: Proteus spp., Providencia stuartii and Morganella morganii are often detected on indwelling catheter surfaces. The presence of these microorganisms leads to many complications such as formation of biofilm encrusted with calcium and magnesium phosphates crystals, which results in catheter blockage (Pallett and Hand, 2010). High ability of urease synthesis is the main reason why Proteus infections cause more cytological damage in renal tissue, than those developed by E. coli (Wilde et al., 2013). This dangerous pathogen, despite the fact that it is often isolated from urological catheters, still remains inadequately characterized. Therefore, the first objective of the investigations presented in this paper was to examine the frequency of P. mirabilis isolation from catheters and assess the complexity of multi-species biofilm formed by these bacteria. The next aim was to determine the susceptibility of P. mirabilis (planktonic and biofilm populations) to antibiotics. Finally, the vulnerability of sessile and planktonic cells of other Gram-negative bacteria (K. pneumoniae, E. coli, Enterobacter cloacae, P. stuartii, M. morganii, P. aeruginosa) was compared to that of P. mirabilis.


table

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strains no.</th>
<th>Gender/patient age</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5</td>
<td>M/72 BPH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>M/92 BPH</td>
<td></td>
</tr>
<tr>
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<td>7</td>
<td>M/77 BPH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>M/83 diabetes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>M/77 BPH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>M/89 BPH</td>
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<td>12</td>
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<td>15</td>
<td>M/82 BPH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>M/86 stroke</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>M/41 multiple sclerosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>M/57 spine damage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>M/80 stroke</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>M/88 BPH, stroke</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>41</td>
<td>M/71 BPH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>M/81 stroke</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>M/81 BPH, stroke</td>
<td></td>
</tr>
<tr>
<td></td>
<td>57</td>
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<td>M/80 BPH</td>
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<tr>
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<td>71</td>
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<td>77</td>
<td>M/67 BPH</td>
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</tr>
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<td>84</td>
<td>M/71 urethral structure</td>
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<td>42</td>
<td>M/72 BPH</td>
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<td>67</td>
<td>M/79 BPH, stroke</td>
<td></td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>31</td>
<td>M/57 spine damage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>W/72 stroke</td>
<td></td>
</tr>
<tr>
<td>Providencia stuartii</td>
<td>9</td>
<td>M/73 BPH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>M/81 stroke</td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>46</td>
<td>M/81 BPH, stroke</td>
<td></td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>M/80 retention of urine</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>11</td>
<td>M/89 BPH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>M/81 BPH, stroke</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
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<td>M/73 BPH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>M/81 stroke</td>
<td></td>
</tr>
</tbody>
</table>

BPH – benign prostatic hypertrophy; M – man; W – woman
pensions were plated on blood and McConkey agars. Next, the identification of morphologically different colonies was performed. The microscopic slide and culturing the bacteria in different identification media were carried out using traditional diagnostic media and API 20 E and NE tests. Antimicrobial sensitivity of *P. mirabilis* planktonic cells was examined by the standard broth-microdilution method by Clinical and Laboratory Standards Institute (CLSI). The MICs (minimum inhibitory concentrations) and MBCs (minimum bactericidal concentrations) were determined in Mueller Hinton broth, by reading the turbidity in wells. Antimicrobials: amoxicillin and clavulanic acid AMC (Polfa), cefazolin CFZ, cephalxin LEX, cefuroxime CXM, cefotaxime CTX, cefaclor CEC, ceftriaxone CRO, gentamicin GM, amikacin AMK (Sigma), ciprofloxacin CIP (Fluka), ofloxacin OFX, norfloxacin NOR (Sigma) and cotrimoxazole SXT (Polfa) were used in the test.

Biofilms susceptibility to antimicrobial agents was studied using the MBEC (minimum biofilm eradication concentration) High-throughput Screening assay (Calgary Alberta, Canada). Antibiotics with the highest activity toward *P. mirabilis* planktonic cells (in the broth-microdilution method) were chosen: amikacin, cephalxin, ceftriaxone; and ciprofloxacin – fluoroquinolone often used in Poland to treat urinary tract infections and the drugs which are not used in the Polish medical practice: cefepime FEP and gatifloxacin GAT (Bristol Squibb Myers).

The test was performed according to the previously described method by Ceri *et al.* (1999), which consists of three stages: 1. Biofilm formation; 2. MIC values determination, 3. MBEC values determination.

### Results

279 microorganisms were isolated from 88 urinary catheter biofilms and classified into 28 species. Over 90% of the isolates were classified into three groups: *Enterobacteriaceae* family – the most frequently isolated bacteria – 53.2%, *Pseudomonadaceae* family – 21.7% and *Streptococcus* group D – 21.3% (Fig. 1A). Distribution of different genera from the *Enterobacteriaceae* family is summarized in Fig. 1B. *E. coli* was the most often isolated species, whereas *Proteus* spp., (17.9% of isolates) was ranked in the third position. Among *Proteus* isolates, *P. mirabilis* species was detected most frequently (88% – 22 strains), mostly from multi-species biofilms (21 cases), composed of 2–7 microorganisms (Table II). Most common *P. mirabilis* associated flora involved: *Streptococcus* group D – in 63% of biofilms and *Pseudomonas* spp. – in 59% of biofilms. *P. stuartii* was often isolated together with *P. mirabilis* strains – almost 50% of *P. stuartii* isolates were acquired from biofilms mixed with *Proteus*.

Thirteen antibiotics were tested against 22 *P. mirabilis* strains. The bacteria were considered sensitive or resistant on the basis of CLSI breakpoints. The reference strain – *E. coli* ATCC 25922 was sensitive to all the tested drugs. The resistance of *P. mirabilis* strains to antibiotics is summarized in Fig. 2. Amikacin had the highest inhibitory and bactericidal activity against *P. mirabilis* strains. 19 out of 22 strains (86%) were susceptible to this drug. High effectiveness of cephalosporins was also detected – 73% (16 strains) of *P. mirabilis* were vulnerable to ceftriaxone, cefotaxime and cefaclor. Fluoroquinolones are
very common antibiotics, which are used to treat UTIs, and ciprofloxacin is administered most frequently. In our study only 8 \textit{P. mirabilis} strains were sensitive to ciprofloxacin and 5 strains were intermediate. Within the fluoroquinolones group – norfloxacin had the best efficiency (70% strains were vulnerable). Cotrimoxazole is usually recommended to treat bacteriuria, but most of the investigated strains were resistant (86%) to this drug.

![Fig. 2. Proteus mirabilis planktonic strains sensitivity to antibiotics: amoxicillin and clavulanic acid (AMC) cefazolin (CFZ), cephalaxin (LEX), cefuroxime (CXM), cefotaxime (CTX), cefaclor (CEC), ceftriaxone (CRO), gentamicin (GM), amikacin (AMK), ciprofloxacin (CIP), ofloxacine (OFX), norfloxacin (NOR) and cotrimoxazole (SXT).]
Among the tested *P. mirabilis* strains, three multidrug resistant strains were found (no. 34, 70 and 84), against which none of the tested antibiotics was active.

The MBEC assay was used to examine the ability of the tested bacteria to form biofilm on polystyrene surface (Table III and IV). All studied uropathogens formed biofilm on polystyrene pegs – *P. mirabilis* with density from 0.04 × 10^6 to 9.91 × 10^6 cells per peg (the most intensive growth was observed for strains no. 70, 71, 46) and in the case of the other uropathogens the average density was, for example, 6.78 × 10^6 CFU/peg for *P. stuartii* and 0.47 × 10^6 CFU/peg for *E. cloacae*.

Six antibiotics were used to study biofilm sensitivity (norfloxacin, ciprofloxacin, amikacin, ceftriaxone, gatifloxacin, cefepime). The distribution of MICs and MBECs is presented in Table III. Significant differences between MICs and MBECs values of most antibiotics were detected. The MBECs were often more than 1000-fold higher than the MICs. The most effective drugs in planktonic cell eradication occurred to be amikacin and ceftriaxone (16 of 22 strains were susceptible). 12 strains were vulnerable to cefepime. Some activity of norfloxacin, ciprofloxacin and gatifloxacin was also observed. Similarly to the results obtained in the microdilution method, three strains were found to be resistant to all the tested antibiotics (*P. mirabilis* 34, 70 and 84). It was revealed that 100% of *P. mirabilis* sessile forms were resistant to norfloxacin, gatifloxacin, cefepime and ciprofloxacin. Amikacin and ceftriaxone inhibited only two *P. mirabilis* strains (5%). The correlation between *P. mirabilis* sessile forms sensitivity to drugs and biofilm intensity was not observed.

*E. coli*, *P. aeruginosa*, *K. pneumoniae*, *P. stuartii*, *E. cloacae* and *M. morganii* were isolated from urinary catheters as *P. mirabilis* associated flora. The susceptibility of these rods to the tested drugs was determined and compared to *P. mirabilis* sensitivity. MICs and MBECs values of the studied uropathogens are presented in Table IV.

The planktonic cells of the uropathogens were mostly vulnerable to the tested drugs e.g. *M. morganii* strains were sensitive to all antibiotics. *K. pneumoniae* strains were resistant only to gatifloxacin; *E. cloacae* to ceftriaxone and ciprofloxacin (strain no. 31). A different observation was made for *P. aeruginosa* strains, which in high concentrations were resistant to all the tested antibiotics.

### Table III

Resistance of *P. mirabilis* planktonic and sessile populations to antibiotics.

<table>
<thead>
<tr>
<th><em>P. mirabilis</em> strains</th>
<th>Inoculum ×10^7 CFU/ml</th>
<th>Biofilm ×10^6 CFU/ml</th>
<th>Resistance of planktonic population (MIC)</th>
<th>Biofilm resistance (MBEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Norfloxacin (NOR); Ciprofloxacin (CIP); Amikacin (AMK); Ceftriaxone (CRO); Gatifloxacin (GAT); Cefepime (FEP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.7</td>
<td>1.77</td>
<td>32</td>
<td>16 &gt;1024 &gt;1024 1024</td>
</tr>
<tr>
<td>6</td>
<td>7.5</td>
<td>0.62</td>
<td>2</td>
<td>4 &lt;1 16 16 &lt;1</td>
</tr>
<tr>
<td>7</td>
<td>5.3</td>
<td>0.23</td>
<td>4</td>
<td>1024 &gt;1024 1024 32</td>
</tr>
<tr>
<td>8</td>
<td>4.8</td>
<td>2.68</td>
<td>32</td>
<td>1024 &gt;1024 1024 32</td>
</tr>
<tr>
<td>9</td>
<td>11.7</td>
<td>0.59</td>
<td>8</td>
<td>1024 &gt;1024 1024 32</td>
</tr>
<tr>
<td>11</td>
<td>4.5</td>
<td>3.05</td>
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<td>1.00</td>
<td>&lt;1</td>
<td>16 &gt;1024 &gt;1024 1024</td>
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<td>6.45</td>
<td>2.83</td>
<td>16</td>
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</tr>
<tr>
<td>31</td>
<td>14.0</td>
<td>2.01</td>
<td>16</td>
<td>1024 &gt;1024 1024 32</td>
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<tr>
<td>32</td>
<td>6.1</td>
<td>1.57</td>
<td>8</td>
<td>1024 &gt;1024 1024 32</td>
</tr>
<tr>
<td>33</td>
<td>10.3</td>
<td>3.91</td>
<td>128</td>
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<td>6.95</td>
<td>9.35</td>
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<td>6.0</td>
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</table>
antibiotics. The most effective drugs against the uropathogens planktonic forms were: amikacin and cefepime.

Biofilms of the studied uropathogens (similarly to those of Proteus strains) were more resistant to the tested antibiotics than planktonic cells (MBECs were 2–512 fold higher than the MIC values), however, the drugs concentrations required for biofilm eradication were lower. Biofilms of M. morganii, E. cloacae and K. pneumoniae strains were more vulnerable to some antibiotics (e.g. M. morganii 42 was sensitive to amikacin, ceftriaxone and gatifloxacin, K. pneumoniae 56 to amikacin, gatifloxacin and cefepime). P. aeruginosa, P. stuartii and E. coli biofilms were resistant to all the tested drugs. There is no connection between biofilm thickness on the pegs and its susceptibility to antimicrobials.

Discussion

Catheter associated UTIs is very common bacterial disease in humans, specially in long time catheterized patients. The cause of this recurrent infections is bacterial biofilm, which coats the catheter surface and constitutes a reservoir of organisms resistant to host defense and drugs. In our research biofilms from 88 catheters, obtained from long term catheterized persons were isolated. About 50% of the isolates belonged to three species of bacteria: Pseudomonas aeruginosa, Enterococcus faecalis and E. coli. Klebsiella pneumoniae and Proteus mirabilis were also commonly detected, with the frequency of 20.7% and 17.9%, respectively. MacLeod and Stickler (2007) examined 106 urinary catheters, collected from hospitalized patients, and isolated a very similar composition of bacterial species (P. aeruginosa, E. faecalis, E. coli, and P. mirabilis). P. mirabilis isolation frequency was higher – 30.2% and that of K. pneumoniae was less frequent (17.9%). When urine specimens collected from long term catheterized or hospitalized patients were analyzed, a different composition of bacterial species was observed: E. coli were the most commonly isolated bacteria (70% of samples), P. aeruginosa – 1.7–4% and Enterococcus spp. – 2–5% of samples (Ghadiri et al., 2012, Niveditha et al., 2012). Proteus isolates were detected with lower frequency (6.7%), which may suggest that Proteus forms crystalline biofilms and bacterial cells are not easily released into urine. Our studies showed that Proteus mirabilis formed multi-species biofilm – 98% of all tested catheters were coated with biofilm consisting of several microorganisms species (2–7 species), only from two catheters single-species biofilm was recovered. Patients, from whom urinary catheters were isolated had been catheterized for a long period of time (from a few months to a few years), time of catheter replacement was different (usually two weeks) – these factors contributed to a number of bacterial species detected in biofilm. By comparison, in Stickler (2008) studies one species of microorganisms was isolated from about 28% of catheters. The species composition of mixed biofilms was diversified, however, P. mirabilis associated flora most often included E. faecalis and P. aeruginosa. We also observed frequent co-colonization of P. stuartii and P. mirabilis. These results confirm the studies described

### Table IV
Resistance of studied uropathogens to antibiotics.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Inoculum ×10⁶ CFU/ml</th>
<th>Biofilm ×10⁶ CFU/ml</th>
<th>Resistance of planktonic population (MIC)</th>
<th>Biofilm resistance (MBEC)</th>
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<td></td>
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<td>NOR</td>
<td>CIP</td>
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<td>4.31</td>
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<td>&lt;1</td>
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<td>0.94</td>
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<td>10.78</td>
<td>16</td>
<td>8</td>
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<tr>
<td>M. morganii 67</td>
<td>12.90</td>
<td>0.70</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>M. morganii 42</td>
<td>12.30</td>
<td>0.60</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>E. cloacae 31</td>
<td>5.40</td>
<td>0.28</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>E. cloacae 36</td>
<td>8.40</td>
<td>0.66</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>E. coli 9</td>
<td>15.20</td>
<td>1.31</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>E. coli 71</td>
<td>12.0</td>
<td>2.20</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

norfloxacin (NOR); ciprofloxacin (CIP); amikacin (AMK); ceftriaxone (CRO); gatifloxacin (GAT); cefepime (FEP)
by MacLeod and Stickler (2007), who also detected Proteus together with E. faecalis and P. stuartii. It is probable that these microorganisms interact in a positive way and form mixed biofilm, which facilitates their survival in the unfavorable urinary tract environment.

It is widely known that P. mirabilis urinary tract infections are difficult to eradicate because these bacilli produce urease, which catalyzes the hydrolysis of urea to ammonia and carbon dioxide. Urea disintegration results in environment alcalization, ammonia contributes to changes in local environment and it is believed to cause damage to host cells (Jacobson et al., 2008, Oki et al., 2010). The consequence of this process is an increase in the urine pH and the production of struvite and carbonate apatite crystals. This process in catheterized patients leads to encrustation and blockage of the catheter lumen – a common problem of long term catheterized persons (Stickler and Feneley, 2011). Wilde et al. (2013) observed that 24% of studied patients reported a few events of catheter blockage in two months, which increased treatment costs, due to the necessity of additional therapy. Interestingly, bladder irrigating (which is not recommended practice and was done by 42% of patients) contributed to catheter blockage (Wilde et al., 2013).

The consequences of P. mirabilis infections are dangerous and it is necessary to devise new, efficient methods, which can help control biofilm related infections. Unfortunately, antibiotics applied in therapy are often ineffective. The National Working Group for Rational Therapy and Prevention of Infections in Poland recommended to treat UTIs in the first place with fluoroquinolones and trimethoprim – sulfamethoxazole. To treat complicated urinary tract infections fluoroquinolones, aminopenicillins with a β-lactamases inhibitor are administered. Aminopenicillins with a β-lactamases inhibitor and cephalosporins are the first drugs prescribed to pregnant women with UTIs (Kupilas, 2006). The studied P. mirabilis strains were resistant to cotrimoxazole, amoxicillin with clavulanic acid (more than 80% of strains). 41% of the studied P. mirabilis strains were also resistant to ciprofloxacin. Ciprofloxacin is characterized by broad activity and reaches a high concentration in blood and UT tissues (Mierzynska and Niemir, 2009). All fluoroquinolones are commonly used to treat UTIs. They could penetrate the biofilm matrix e.g. ciprofloxacin penetrates into the biofilm structure within 20 minutes and obtains the MIC (Anderl et al., 2000). Furthermore, these antibiotics are active against slow growth cells so they are recommended to treat biofilm related infections (Passerini de Rossi et al., 2009).

Cephalosporins, very often recommended to treat UTIs, have great bactericidal activity with the exception of strains producing ESBL (Extended-Spectrum Beta-Lactamases). 14.4% of P. mirabilis strains in Poland produce ESBL (Kwiecinska-Piróg et al., 2010). It has been proved that Proteus strains, synthesizing these enzymes and forming strong biofilm, are particularly dangerous and infections caused by them are persistent and difficult to treat (Nucleo et al., 2010). Most studied strains (planktonic populations) were vulnerable to these drugs, but their biofilms were resistant. Explanation for biofilm resistance could be the presence of β-lactamases, which are accumulated in the biofilm matrix – β-lactams antibiotics could be hydrolyzed before their penetration into biofilm (Hoiby et al., 2010). Spoering and Lewis (2001) suggest that biofilm resistance could result from the fact that microorganisms in biofilm have slow metabolism, similarly to bacteria in the stationary growth phase, while β-lactams are antibiotics which bind to fast growing cells. It is possible that cephalosporins (e.g. cefuroxime) activity relies only on delaying biofilm formation (Koseoglu et al., 2006, Spoering and Lewis 2001). Cefepime is characterized by stability against ESBL and AmpC enzymes and it is recommended to treat UTIs caused by ESBL-producing organisms (da Silva Nogueira et al., 2011). Bantar et al. (2004) suggest that substituting ceftriaxone with cefepime in UTIs caused by P. mirabilis results in a decrease in bacterial resistance to cephalosporins.

Aminoglycosides are recommended as an alternative to fluoroquinolones and cephalosporins to treat especially complicated UTIs (Kupilas, 2006). However, amikacin is not often used to treat UTIs because of dangerous side effects, despite the fact that its concentration in kidneys is high. The examined P. mirabilis planktonic cultures occurred to be sensitive to amikacin. The rare administration of this drug (usually in hospitals) in UTIs could be the reason for its high efficacy in P. mirabilis eradication. P. mirabilis biofilms were more resistant to this antibiotic contrary to biofilms of the other studied uropathogens (M. morganii, E. cloaca, K. pneumoniae). The extracellular matrix binds the drugs and inhibits penetration to deeper biofilm layers, which could be the reason why biofilms are resistant to aminoglycosides (Spoering and Lewis 2001). It is also probable that the oxygen limitation and the metabolic rate play an important role in this process (Hoiby et al., 2010).

Because of a higher rate of bacterial resistance to popular drugs, many urologists increasingly suggest a change of bad antibiotic-prescription behaviour. Trimethoprim/sulfamethoxazol and fluoroquinolones should not be recommended for therapy in the case of complicated UTIs (Mazzulli, 2012). Fosfomycin/trometamol, nitrofurantoin, pivmecillinam should be current drugs of first choice (Wagenlehner and Naber, 2012). Pallett and Hand, 2010 also suggest alternative treatment e.g. combination of ceftiofurine or cefpirome with clavulanate or fosfomycin.
In conclusion, *P. mirabilis* is very often isolated from urinary catheters, where it forms encrusted, multi-species biofilm, resistant to the majority of drugs. Biofilm related infections are persistent and difficult to treat. It is important to administer antibiotics with care (e.g. appropriate drugs in adequate doses), because the consequence of badly conducted treatment could be the extension of bacterial resistance to antibiotics.

**Acknowledgments**

We would like to thank Bristol-Myers Squibb for providing cefepime and gatifloxacin samples and allowing us to use them in our study.

**Literature**


**Introducción**

Las infecciones del tracto respiratorio superior (URTIs) son las enfermedades más comunes durante el periodo infancial, pero su origen clínico no puede ser diferenciado en función de la etiología viral o bacteriana. Como causa importante de visitas a los médicos de familia y como razón clave de prescripción de antibióticos, son de especial relevancia.

*Streptococcus pneumoniae* es un importante agente etiológico de meníngeas, neumonias, bacteriemias y otitis media aguda, tanto en niños como en adultos y uno de los agentes colonizadores más importantes del nasofaringe, principalmente asintomáticamente (Bogaert *et al.*, 2004). Los niños pequeños son colonizados con mayor frecuencia y se han identificado como reservorios principales de *S. pneumoniae*, jugando un rol crucial en la propagación y selección de cepas resistentes a múltiples medicamentos (De Lencastre y Tomasz, 2002). Recientemente, se ha visto una tasa cada vez mayor de resistencia a antibióticos en *S. pneumoniae*, particularmente entre los cepas de *S. pneumoniae* (2002). Antibiotico resistente *S. pneumoniae* se distribuyen principalmente entre serotypes de *S. pneumoniae*. Buen cobertura de vacunas con serotypes de *S. pneumoniae* confirmó que la introducción de PCVs en el programa de vacunación del país puede reducir la población de resistencia y resistencia a múltiples medicamentos de *S. pneumoniae* en la comunidad.

**Palabras clave**: *Streptococcus pneumoniae*, adenoids, antibiotic resistance patterns, serotypes
children under 2 years old but not refunded by health authorities. Since 2009, the PCV was recommended for children under 5 years old and refunded for some risk groups. According to our previous study, the adenoids like the nasopharynx can be regarded as a reservoir of \textit{S. pneumoniae}, including resistant and/or multiresistant strains. As a continuation of that study we investigated pneumococci isolated from adenoid tissue of children aged 2–5, who had gone adenoidectomy for recurrent and/or persistent symptoms of upper respiratory tract infections. Serotypes and antibiotic resistance patterns of the isolated pneumococci were determined and also risk factors of adenoid colonization by pneumococci were defined.

**Experimental**

**Material and Methods**

**Patients.** The study enrolled 103 children, aged between 2 and 5, undergoing adenoidectomy in Department of Pediatric Otolaryngology, Phoniatrics and Audiology, Medical University of Lublin during May-June and November-December 2011. The indication for adenoidectomy was recurrent acute pharyngotonsilitis for at least 2 years with 5 or more acute attacks per year. Patients didn't receive any antibiotic therapy for at least 20 days before the operation. From all children's parents, the informed content were obtained. The Ethical Committee of the Medical University of Lublin approved the study protocol (No. KE-0254/75/211).

Demographic data of studied children was shown in Table I. None of the children were immunized by a pneumococcal vaccine.

**Laboratory procedures.** After the surgery, the adenoid were placed in the sterile container and were transported to laboratory then the adenoid was swabbed with sterile alginate-tipped applicator. Swabs were inoculated on selective Mueller-Hinton agar with 5% sheep blood and 0.5 mg/L of gentamicin for selective cultivation of streptococci. The streaked agar plates were incubated aerobically at 35°C in 5% CO\textsubscript{2} enriched atmosphere for 24 to 48 hours. Pneumococci were identified by colony morphology, susceptibility to optochin (5 µg), and bile solubility; identification was confirmed by a slide agglutination test Slidex Pneumo-Kit (BioMerieux).

All isolates were serotyped by means of Quellung reaction using antisera provided by Statens Serum Institute (Copenhagen, Denmark). We applied antisera for determination of serotypes belonging to the 23-valent pneumococcal polysaccharide vaccine (PPV23), \textit{i.e.} – 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F) and also serotypes 6A, 23A. The isolates negative to employed pooled sera but positive to omni serum were defined as Others, serotypes not present in PPV23. The isolates negative to slide agglutination test and negative to omni serum were defined as untypeable (rough – R); their belonging to pneumococci was confirmed by PCR analysis using primers for detecting the \textit{lytA} gene encoding the autolysin enzyme specific to \textit{S. pneumoniae} (Simoes et al., 2011).

![Table I](image)

Demographic data of children undergoing adenoidectomy for recurrent URTIs.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Total number of children (%)</th>
<th>SP colonized children (% in group)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (yr)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11 (10.7)</td>
<td>6 (54.5)</td>
</tr>
<tr>
<td>3</td>
<td>24 (23.3)</td>
<td>11 (45.8)</td>
</tr>
<tr>
<td>4</td>
<td>51 (49.5)</td>
<td>33 (64.7)</td>
</tr>
<tr>
<td>5</td>
<td>17 (16.5)</td>
<td>12 (70.6)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>43 (41.7)</td>
<td>26 (60.5)</td>
</tr>
<tr>
<td>male</td>
<td>60 (58.3)</td>
<td>36 (60.0)</td>
</tr>
<tr>
<td><strong>Sibling possessing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>17 (16.5)</td>
<td>11 (64.7)</td>
</tr>
<tr>
<td>1</td>
<td>46 (44.6)</td>
<td>28 (60.9)</td>
</tr>
<tr>
<td>≥ 2</td>
<td>40 (38.8)</td>
<td>23 (57.5)</td>
</tr>
<tr>
<td><strong>Passive smoking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>32 (31.1)</td>
<td>19 (59.4)</td>
</tr>
<tr>
<td><strong>DCC attendance</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>83 (80.6)</td>
<td>49 (59.0)</td>
</tr>
<tr>
<td><strong>Place of residence</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>village</td>
<td>41 (39.8)</td>
<td>23 (56.1)</td>
</tr>
<tr>
<td>city</td>
<td>62 (60.2)</td>
<td>39 (62.9)</td>
</tr>
<tr>
<td><strong>Antibiotics taken</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>for the last attack</td>
<td></td>
<td></td>
</tr>
<tr>
<td>amoxicillin/co-amoxiclav</td>
<td></td>
<td>55 (54.5)</td>
</tr>
<tr>
<td>cephalosporins</td>
<td>24 (23.8)</td>
<td>16 (66.7)</td>
</tr>
<tr>
<td>macrolides</td>
<td>22 (21.8)</td>
<td>10 (45.5)</td>
</tr>
</tbody>
</table>

DCC, day care center; SP, \textit{Streptococcus pneumoniae}
Susceptibility of the isolates to oxacillin, erythromycin (E), tetracycline (Te), chloramphenicol (C), clindamycin (Cc), norfloxacain (Nor), rifampicin (Ra), teicoplanin (Tec), linezolid (Lzd) and trimethoprim-sulfamethoxazole (Sxt) was determined by the disk diffusion method of Bauer and Kirby. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing recommendations (EUCAST, 2011). Isolates exhibiting a zone of ≥20 mm around a 1 µg oxacillin disk were reported as penicillin susceptible *S. pneumoniae* (PSSP); isolates exhibiting a zone of <20 mm were further tested by the E-test (AB Biodisk, Sweden), following the manufacturer’s instruction, to determine minimal inhibitory concentration (MIC) for benzylpenicillin. Isolates with MIC ≤0.064 mg/L were considered as fully susceptible to benzylpenicillin; isolates with MIC >0.064 mg/L were called penicillin non-susceptible *S. pneumoniae* (PNSSP). Multidrug-resistant isolates of *S. pneumoniae* (MDR-SP) were defined as having resistance to at least 3 different classes of antibiotics. *S. pneumoniae* ATCC 49619 was used as control strain in the antimicrobial susceptibility tests.

**Statistical analysis.** Data processing and analysis were performed using StatSoft, Inc. STATISTICA 10. The potential predictor variables were tested in separate univariate analyses (Chi-squared or the Fisher exact test, as appropriate) for their association with upper respiratory colonization by *S. pneumoniae* in general, and by PNSSP or MDR-SP. Significant univariate predictors (p<0.1) were tested for inclusion in the multivariate models, and nonsignificant variables were removed sequentially until only those significant at p<0.1 remained. Variables of particular interest based on previous studies, such as children age, having siblings, passive smoking and type of antibiotic used, were included even when not statistically significant. Statistical significance was set at p<0.05.

**Results**

*S. pneumoniae* colonization was observed in 62 (60.2%) children who had undergone adenoidectomy for recurrent and/or persistent symptoms of upper respiratory tract infections. A total of 66 isolates were recovered; 4 (3.9%) children were colonized by two different in colony morphology isolates, which were identified by serotyping and antimicrobial resistance tests as different pneumococcal strains. Among the isolates, serotypes belonged to PPV23 (89.4%) were identified, and 4 isolates (6.1%) were untypeable (rough – R) (Table II).

The most frequent was serotype 19F (24.2%). Serotypes belonged to pneumococcal conjugated vaccines

<table>
<thead>
<tr>
<th>Serotypes</th>
<th>No. (%) of isolates</th>
<th>Antibiotic resistance pattern (no. of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5 (7.6)</td>
<td>S (5)</td>
</tr>
<tr>
<td>6A</td>
<td>2 (3.0)</td>
<td>ECcTeSxt (1), S (1)</td>
</tr>
<tr>
<td>6B</td>
<td>9 (13.6)</td>
<td>PECcCTeSxt (2), PECcCSxt (4), PECcTeSxt (1), S (2)</td>
</tr>
<tr>
<td>9V</td>
<td>2 (3.0)</td>
<td>Psxt (2)</td>
</tr>
<tr>
<td>10A</td>
<td>1 (1.5)</td>
<td>CTe (1)</td>
</tr>
<tr>
<td>11A</td>
<td>3 (4.5)</td>
<td>S (3)</td>
</tr>
<tr>
<td>14</td>
<td>2 (3.0)</td>
<td>PECcTeSxt (1), PECcSxt (1)</td>
</tr>
<tr>
<td>15B</td>
<td>2 (3.0)</td>
<td>Sxt (1), S (1)</td>
</tr>
<tr>
<td>15 (nonB)</td>
<td>4 (6.1)</td>
<td>Sxt (2), S (2)</td>
</tr>
<tr>
<td>18C</td>
<td>1 (1.5)</td>
<td>S (1)</td>
</tr>
<tr>
<td>19A</td>
<td>1 (1.5)</td>
<td>PECcTeSxt (1)</td>
</tr>
<tr>
<td>19F</td>
<td>16 (24.2)</td>
<td>PECcCTeSxt (4), PECcTeSxt (4), ECcCTeSxt (1), ECcTeSxt (2), ECcTe (1), CTeSxt (1), CSxt (1), S (2)</td>
</tr>
<tr>
<td>23A</td>
<td>1 (1.5)</td>
<td>ECcCTe (1)</td>
</tr>
<tr>
<td>23B</td>
<td>1 (1.5)</td>
<td>S (1)</td>
</tr>
<tr>
<td>23F</td>
<td>7 (10.6)</td>
<td>PECcCTeSxt (1), PECcCSxt (4), PECcTeSxt (1), Sxt (1)</td>
</tr>
<tr>
<td>33F</td>
<td>2 (3.0)</td>
<td>ECcTe (2)</td>
</tr>
<tr>
<td>Others</td>
<td>3 (4.5)</td>
<td>PECcTeSxt (1), S (2)</td>
</tr>
<tr>
<td>R</td>
<td>4 (6.1)</td>
<td>PECcTeSxt (3), PCSxt (1)</td>
</tr>
</tbody>
</table>

P, penicillin; E, erythromycin; Cc, clindamycin; Te, tetracycline; C, chloramphenicol; Sxt, co-trimoxazol; S, sensitive to all tested antibiotics; R, rough, untypeable strain; Others, serotypes not present in PPV23.
– PCV10 (containing serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F) and PCV13 (containing serotypes 3, 6A, 19A additionally to 10-valent vaccine) constituted 56.1% and 68.2% of the isolates, respectively.

The pneumococcal isolates were susceptible to all tested antimicrobial agents in 30.3%. These strains belonged to serotypes 3 (5 isolates), 11A (3 isolates), 6B, 19F, 15 non-B, NT (2 isolates per each serotype), and 6A, 10A, 15B, 18C (1 isolate per each serotype). Among all of the strains, 45.5% had decreased susceptibility to penicillin (MIC range 0.12–2.0 mg/L, MIC₉₀ 0.5 mg/L and MIC₅₀ 2.0 mg/L). S. pneumoniae isolates were resistant to co-trimoxazole (62.1%), tetracycline (43.9%), erythromycin (54.5%), clindamycin (54.5%) and chloramphenicol (31.8%) (Fig. 1). All isolates were susceptible to norfloxacin and according to EUCAST 2011, they should be reported as susceptible to levofloxacin and moxifloxacin and intermediate to ciprofloxacin and ofloxacin. None of the tested isolates was resistant to rifampicin, linezolid and teicoplanin. Each strain was characterized phenotypically by serotype and antibiotic resistance pattern (Table II). Multidrug resistance was present in 57.6% of isolates. Among MDR-SP 76.3% were non-susceptible to penicillin. Antibiotic resistant pneumococci were mostly distributed among serotypes belonged to PCV10 and PCV13 (Fig. 1). PNSSP and MDR-SP strains represented PCV10 serotypes in 83.3% and 73.6%, respectively and PCV13 serotypes in 86.7% and 78.9%, respectively. Colonization with PNSSP and MDR-SP strains was found in 30 (29.1%) and 35 (34.0%) children, respectively.

During the analysis of risk factors predisposing to pneumococcal colonization, including PNSSP and MDR strains, no predictors were found in the total population of children. However, when analysis in group of 32 children exposed to passive smoking was done, two predictors turned out to be significant for pneumococcal colonization: female gender (p = 0.049, OR 1.59, 98%CI 1.1–2.4) and type of antibiotics taken for the last attack (p = 0.019). Moreover, in these children consumption of β-lactams increased the risk of pneumococcal colonization in comparison to macrolide consumption (p = 0.015, RR 5.1, 95%CI 1.2–20.8). Type of taken antibiotics was a predictor of MDR-SP colonization in children exposed to passive smoking (p = 0.02). Macrolide consumption in these children decreased rate of MDR-SP colonization in comparison to children with consumption of β-lactams (p = 0.012, RR 0.55, 95%CI 0.4–0.8).

**Discussion**

The adenoids are involved in the pathology of adenotonsillitis, rhinosinusitis, otitis media and chronic nasal obstruction (Brook and Shah, 2001; Karlidag et al., 2002). High frequency of adenoid colonization by S. pneumoniae in preschool children with recurrent upper respiratory infections was found in our study, which is in agreement with the previous observations (Niedzielski et al., 2013). However, high rate of asymptomatic pneumococcal carriage in preschool children, especially attending day care center is common so it is difficult to indicate that S. pneumoniae is an important bacterial etiological agent of URTIs even though it is isolated from children with URTI symptoms (Gunnarsson et al., 2001). Jeong et al. (2007) analyzed the differences between the bacterial pathogens of tonsillar core in recurrent tonsillitis and tonsillar hypertrophy with regard the age; S. pneumoniae was detected with high frequency in recurrent tonsillitis in the patients between 8–14 years, even though this pathogen was more common in younger patients (<8 years) in both groups. Syrjanen et al. (2001) reported that nasopharyngeal carriage of pneumococci during RTIs (without otitis media) in children increased from 13–43% to 45–56%, depending on age. Contrary Greenberg et al. (2004) found no differences in the overall S. pneumoniae carriage between healthy and sick children in different age groups.

Resistant pneumococci are common among young children, especially attending day care centers. Transmission of these resistant strains in the community is constantly investigated (De Lencastre and Tomasz, 2002; Borer et al., 2001). Resistance to antimicrobial agents among pneumococcal isolates in this study was much higher than data concerning pneumococcal isolates described in Poland (Jacobs et al., 2003; Riedel...
et al., 2007). Children with recurrent pharyngotonsillitis are usually treated with multiple courses of antibiotics before surgery but many of them continue to carriage of pathogenic bacteria in the pharynx and the adenoids, including strains resistant to antibiotic (McCay, 2000) and all of children from our studies were treated by antibiotics before surgery. Poland belong to a part of Europe with high level of antibiotic consumption and above 20% rate of penicillin and macrolide resistance among the pneumococcal isolates (Riedel et al., 2007, van de Sande-Bruinsma et al., 2008).

The passive smoking increases colonization rate by pathogens in children and their risk for respiratory tract infections (Gryczynska et al., 1999; Kosikowska et al., 2011). Principi’s et al. (1999) observation with healthy children indicated that exposure to tobacco smoke did not influence the *S. pneumoniae* carriage in the upper respiratory tract in children aged <5 years. Bakhshaee et al. (2012) found a significant difference in carriage rates between children who lives in smoking families compare to those with nonsmoking families for *M. catarrhalis*, but not for *S. pneumoniae* and *H. influenzae*. However, Greenberg et al. (2006) showed that exposure to tobacco smoke increased *S. pneumoniae* carriage rates particular in children. Data obtained in the present study indicated no influence of passive smoking on pneumococcal colonization in children with recurrent URTIs. However, contrary to our previous data (Niedzielski et al., 2013), in present paper no risk factor for pneumococcal colonization in total population of children undergoing adenoidectomy for recurrent URTIs was found, but in group of children exposed to passive smoking some predictors of the adenoid colonization by pneumococci were revealed – female gender as a predictor of pneumococcal colonization and type of antibiotics taken for last attack as a predictor of pneumococcal colonization and MDR-SP colonization. These observations suggest that the effect of passive smoking on pneumococcal prevalence in the upper respiratory tract, including adenoids, may be correlated with other predisposing factors, e.g. age, gender, season, staying in a close population (e.g. day care center), chronic respiratory infections, previous antibiotic treatment, allergy or geographic region.

Our data revealed that consumption of β-lactams increased the rate of pneumococcal colonization in adenoids but only in children exposed to tobacco smoke. On the other side the decrease of MDR-SP colonization was observed in this group of children who received macrolides. Some studies demonstrated an association between the use of a specific antibiotic and selective colonization with strains resistant to this drug as well as that number of courses of drugs to which patho-

gastric bacteria in the pharynx and the adenoids, including strains resistant to antibiotic (McCay, 2000) and all of children from our studies were treated by antibiotics before surgery. Poland belong to a part of Europe with high level of antibiotic consumption and above 20% rate of penicillin and macrolide resistance among the pneumococcal isolates (Riedel et al., 2007, van de Sande-Bruinsma et al., 2008).

The passive smoking increases colonization rate by pathogens in children and their risk for respiratory tract infections (Gryczynska et al., 1999; Kosikowska et al., 2011). Principi’s et al. (1999) observation with healthy children indicated that exposure to tobacco smoke did not influence the *S. pneumoniae* carriage in the upper respiratory tract in children aged <5 years. Bakhshaee et al. (2012) found a significant difference in carriage rates between children who lives in smoking families compare to those with nonsmoking families for *M. catarrhalis*, but not for *S. pneumoniae* and *H. influenzae*. However, Greenberg et al. (2006) showed that exposure to tobacco smoke increased *S. pneumoniae* carriage rates particular in children. Data obtained in the present study indicated no influence of passive smoking on pneumococcal colonization in children with recurrent URTIs. However, contrary to our previous data (Niedzielski et al., 2013), in present paper no risk factor for pneumococcal colonization in total population of children undergoing adenoidectomy for recurrent URTIs was found, but in group of children exposed to passive smoking some predictors of the adenoid colonization by pneumococci were revealed – female gender as a predictor of pneumococcal colonization and type of antibiotics taken for last attack as a predictor of pneumococcal colonization and MDR-SP colonization. These observations suggest that the effect of passive smoking on pneumococcal prevalence in the upper respiratory tract, including adenoids, may be correlated with other predisposing factors, e.g. age, gender, season, staying in a close population (e.g. day care center), chronic respiratory infections, previous antibiotic treatment, allergy or geographic region.

Our data revealed that consumption of β-lactams increased the rate of pneumococcal colonization in adenoids but only in children exposed to tobacco smoke. On the other side the decrease of MDR-SP colonization was observed in this group of children who received macrolides. Some studies demonstrated an association between the use of a specific antibiotic and selective colonization with strains resistant to this drug as well as that number of courses of drugs to which pathogens are resistant has the utmost importance (Appelbaum, 2002). Borer et al. (2001) described statistically significant impact of antimicrobial drug use on nasopharyngeal carriage of *S. pneumoniae*, whereas Principi et al. (1999) showed macrolide therapy increased nasopharyngeal pneumococcal carriage. Findings from southern Israel strongly suggest that azithromycin affects increased multidrug resistance in *S. pneumoniae* (Barkai et al., 2005).

Widespread use of PCVs resulted in decreased in invasive pneumococcal disease and pneumonia among children and elderly persons in countries that have introduced conjugate vaccines (Center, 2008; Lopalco, 2009). In Poland PCV is already included to routine immunization program: vaccination is recommended universally for children ≤ 2 years old and since 2009 is mandatory and refunded for some risk groups. However, low vaccination rate was observed, especially in south-east region of Poland. Our data showed that the *S. pneumoniae* serotype coverage of isolates colonizing adenoids in pre-school children with recurrent/ persistent URTIs from Poland by the currently available PCVs is high (56.1–68.2%) and similar to that reported in other European countries (McIntosh et al., 2007). According to studies performed by Skoczynska et al. (2011), in children aged less than 5 years in Poland, serotypes 14, 6B, and 19F were most prevalent, comprising 52.7% of the invasive pneumococcal disease (IPD) cases. The PCV10, and PCV13 covered 54.8%, and 68.8% of all IPD cases, and 76.3%, and 86.3% of cases involving children under 5 years of age. An encouraging finding of our present data was also that a majority of PNSSP and MDR-SP belonged to serotypes included in PCV13 and PCV10. The above data suggest that routine vaccination of infants with PCVs could effectively reduce the reservoir of pneumococci within upper respiratory tract, including resistant and/ or multidrug resistant strains, in children in Poland, similarly to that in other European countries (O’Brien et al., 2009).

High carriage rate of *S. pneumoniae* in adenoids, including multidrug resistant strains, was observed in our study in children with an indication for adenoidectomy due to recurrent upper respiratory tract infections refractory to antibiotic therapy. Good vaccine coverage among the isolated pneumococci allows the suggestion that the introduction of PCVs in the national immunization program in young children may reduce the high carriage rate of pneumococci, colonizing not only nasopharynx but also adenoids.

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Literature


Iron (Fe) is the fourth most abundant element in the Earth’s crust, and the second most abundant element that is redox-active in near-surface aqueous habitats, providing it the most vital metal in the environment (Cornell and Schwertmann, 1996; Edwards et al., 2004). The largest source of iron in the oceans is probably the atmosphere and this comes mainly from the wind erosion of soils to form dust. Iron is involved in many key biological processes like photosynthesis, N₂ fixation, methanogenesis, H₂ production and respiration, oxygen transport, gene regulation and DNA biosynthesis (Andrews et al., 2003). Microorganisms are involved in the precipitation and solubilization of iron by growing them at the expense of energy gained from the oxidation of reduced iron or by reduction of ferric ions (Cameron et al., 1984). Iron-oxidizing bacteria (FeOB) have been noticed in a wide range of environments and exposed to increase the rate of Fe oxidation by up to four orders of magnitude compared with the rate of strictly abiotic oxidation (Sogaard et al., 2001). FeOB are important catalysts of Fe cycling; on the other hand little is known about their diversity and distribution in various environments (Wang et al., 2009). Secretion of siderophores, the iron solubilizing material produced by the marine bacteria to facilitate the iron uptake into microbial cells has been reported by Trick (1989) and Okujo et al. (1994). Marine bacteria hold more iron per biomass than phytoplankton, the major primary producers (Tortell et al., 1996). For instance the bacterium Thiobacillus ferrooxidans gains energy from ferrous ion oxidation, but a wide variety of bacteria may deposit ferric iron without necessarily obtaining energy from the process.

Most of the iron in the seawater is present in the oxidation state Fe(III) which in alkaline conditions of sea should readily form insoluble iron oxides (Turner and Hunter, 2001). Although heterotrophic bacteria require up to one micro molar iron for growth, the total amount of iron in surface ocean water is sub nano molar. Dissolved iron level in open ocean water is about 20 pmol – 1 nmol/l (Wu and Luther, 1994), which is much lower than the concentration required for most of the microorganisms for their growth. This limiting amount of iron has implications in the biogeochemical cycling of iron.
carbon and in limiting phytoplankton growth. Marine bacteria can successfully compete for this limited nutrient using a specialized iron transport system, including the production and release of siderophores. Chemically siderophores are low molecular weight metabolites with masses of < 2000 Da that have high affinity for ferric ion (Schalk et al., 2011). The role of these compounds is to scavenge iron from the environment and to make the mineral which is almost always essential, available to the microbial cell (Reid and Butler 1991; D’Onofrio et al., 2010). In this study we planned to investigate the culturable fraction of iron bacterial diversity from the Arabian Sea (AS) and Equatorial Indian Ocean (EIO) which are of having moderate and low iron concentrations to compare and to understand how these two different environments fulfill the iron requisite of the bacteria present over.

Experimental

Materials and Methods

Sampling details. EIO Sampling was carried out during the cruise track of Boris Petrov ABP#37 from June 09 to July 10, 2009; stations were occupied at one degree intervals across the equator along 83°E from 1°N to 5°S. Fifty five sediment samples and 98 water samples were collected and analyzed. AS water and sediment samples were collected at different locations of Off Goa during the cruise by coastal research vessel Sagar Suktı (SASU#185, August 2009) along 15°N between 72 and 73°E.

Isolation of iron bacteria. Iron Bacteria were isolated using the media M622- HiMedia, Mumbai (g/l: Glucose 0.15, Ammonium sulphate 0.5, Calcium nitrate 0.01, Dipotassium phosphate 0.05, Magnesium sulphate 0.05, Potassium chloride 0.05, Calcium carbonate 0.1, Vitamin B12 0.00001, Thiamine 0.0004, Agar 10.0). Appropriately diluted samples were spread plated on Iron bacterial isolation medium prepared in 50% seawater. The bacterial cultures were spot inoculated in Yeast extract – 1.0 and Agar – 20.0 prepared in 50% seawater. The medium and incubated for 24–72 h. Appearance of Y orange/yellow zone around the colony indicated siderophore production.

Screening of siderophore producing bacteria. To screen the low molecular-weight Fe(III) specific ligands (siderophores), Chrome azurol sulphonate (CAS) plate method was used (Schwyn and Neilands, 1987) and the media contains (g/L) CAS – 0.726; Hexadecyltrimethylammonium bromide (HDTMA) – 0.1456; Iron solution (1 mM FeCl3, 10 mM HCl) – 2 mL; Peptone – 5.0; Yeast extract – 1.0 and Agar – 20.0 prepared in 50% seawater. The bacterial cultures were spot inoculated in the medium and incubated for 24–72 h. Appearance of orange/yellow zone around the colony indicated siderophore production.

Characterization of siderophores. Cell free culture supernatants were examined for various types of siderophores i.e., hydroxamate nature by FeCl3 and tetrazo-
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Quantification of siderophores. Quantitative estimation of siderophores was done by CAS-shuttle assay (Payne, 1994). In which 0.5 mL of culture supernatant was mixed with 0.5 ml of CAS reagent, and absorbance was measured at 630 nm against a reference consisting of 0.5 ml of uninoculated broth and 0.5 ml of CAS reagent. Siderophore content in the aliquot were calculated by using the formula: % siderophore units = (Ar-As)/Ar (Where, Ar = absorbance of reference at 630 nm (CAS reagent) and As = absorbance of sample at 630 nm).

UV-Visible spectrophotometer scanning. Bacterial cultures were grown in TYES medium (g/l: Tryptone 4.0, Yeast Extract 0.04, CaCl₂·2H₂O 0.2, MgSO₄·7H₂O 0.5, Glucose 0.05) for 6 days at 30°C. The cells were removed by centrifugation at 6000 × g for 15 min. The pH of the supernatant were adjusted to 2.0 with 12N HCl and extracted with 0.4 volume of ethyl acetate using separating funnel. The extracts were then concentrated by using rotary vacuum evaporator (Roteva, Equitron). Dried sample was re-suspended with 1 ml of methanol and subjected to scan from 200–800 nm under UV-Visible spectrophotometer (UV-2450, Shimadzu). Base line was corrected with the solvent methanol.

Results and Discussions

A total of 92 bacterial colonies were isolated from the iron media based on morphological characteristics like colour, size, shape, texture, Gram’s and spore staining. Most of the colonies were circular, white or dull white and only few were accounted for yellow. Around 95% of the isolates were rod shaped and very few were in coccid nature (Fig. 1). Seventy percent of the EIO isolates were gram positive, spore producers and in contrast 92% of the AS isolates were gram negative (data not shown).

Approximately 1500 nucleotides long PCR amplified 16S rDNA of the bacterial isolates were differentiated for its phylotypes using ARDRA analysis with Alu-I endonuclease (AG↓CT). ARDRA of the culturable iron bacterial isolates sorted them into various phylotypes (39 restriction patterns) which were then sequenced. Identifications based on 16S rRNA gene sequence comparison to BLAST analysis of the iron bacteria are shown in Table I. Chimera check indicated that there were no anomalies detected from the sequences. The 16S rRNA gene sequences expressed three different bacterial classes, each one represented by different families: Bacilli (Bacillaceae and Staphylococcaceae), α-Proteobacteria (Rhodobacteraceae, Erythrobacteraceae and Phyllobacteriaceae) and γ-Proteobacteria (Pseudoalteromonadaceae, Alteromonadaceae, Alcanivoracaceae, Pseudomonadaceae, Idiomarinaceae, Chromatiaceae and Halomonadaceae). 16S rRNA gene sequencing analysis exhibited 13 different genera belong to Halomonas, Rheinheimera, Staphylococcus, Marinobacter, Idiomarina, Alcanivorax, Erythrobacter, Roseovarius, Sagittula, Nitratireductor, Pseudoalteromonas, Pseudomonas and Bacillus (Table I).

Iron bacterial 16S rRNA gene similarity levels were mostly ≥97% when compared with the published
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GenBank database. But some of the Arabian Sea isolates like ASW4a and ASS5a were matched only 96.8% with the existing database. Since the similarity indexes are showing lower than 97%, this could expect to form a new species under the genera *Pseudoalteromonas*. Further one of the isolate ASS2A from the Arabian Sea observed the similarity level of 94.7% may form a potential new genus in the family Pseudoalteromonadaceae. Additional experiments like Fatty Acid Methyl Ester analysis and DNA-DNA relatedness have to be done to confirm this. Even though more than 97% similarity level were observed for some of the Indian Ocean isolates like OW6, OW10, OW11, OW12, OW18, OS1B, OS1C and OS42, these species were reported very rarely in the literatures. Above results clearly indicate many novel and rare bacterial species on Iron bacteria to be included in the diversity data bases.

Much less information is available about iron oxidizing bacteria from the marine environment because their habitats appear limited primarily to deep sea

![Fig. 2. The Neighbor-Joining tree constructed based on evolutionary distances and computed using the Maximum Composite Likelihood method representing relationship between the 16S rRNA sequence of iron bacteria (a. Equatorial Indian Ocean; b. Arabian Sea). Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the tree branch points. *Anaerolinea thermolithosa* is used as an out group. Bacteria with accession numbers provided in the phylogenetic tree were retrieved from GenBank database to know the relationship of our bacteria.](image-url)
associated with hydrothermal activity or rendered ocean crust that are difficult to study. Gallionella fer-
ruginea, Leptothrix spp., Sideroxydans spp., Mariprofun-
dus ferrooxydans and Thiobacillus ferrooxidans were
well documented iron oxidizing bacteria isolated from
various aquatic environments (Emerson et al., 2010).
On the other hand Pseudoalteromonas, Pseudomonas,
Vibrio, Halomonas, Marinobacter, Shewanella and Idio-
marina are some of the other organisms which could
do better iron oxidation (Sudek et al., 2009). Present
investigation conducted from the AS and EIO were
also shown that the heterotrophic organisms (Table I)
belongs to γ- and α-Proteobacteria were able to per-
form better iron oxidation than the traditional ones

Table I
16S rRNA gene identity of iron bacteria from EIO and AS.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Strain no.</th>
<th>Bacteria identified by 16S rRNA gene</th>
<th>Phyla/Class</th>
<th>Accession no.</th>
<th>Similarity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OW1</td>
<td>Marinobacter flavimaris</td>
<td>γ-Proteobacteria</td>
<td>JQ905060</td>
<td>99.6</td>
</tr>
<tr>
<td>2</td>
<td>OW3</td>
<td>Bacillus flexus</td>
<td>Bacilli</td>
<td>JQ905061</td>
<td>99.2</td>
</tr>
<tr>
<td>3</td>
<td>OW6</td>
<td>Alcanivorax venustensis</td>
<td>γ-Proteobacteria</td>
<td>JQ905062</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>OW9</td>
<td>Erythrobacter flavus</td>
<td>α-proteobacteria</td>
<td>JQ905063</td>
<td>99.9</td>
</tr>
<tr>
<td>5</td>
<td>OW10*</td>
<td>Marinobacter hydrocarbonoclasticus</td>
<td>γ-Proteobacteria</td>
<td>JQ905064</td>
<td>97.7</td>
</tr>
<tr>
<td>6</td>
<td>OW11</td>
<td>Roseovarius rubinhibens</td>
<td>α-Proteobacteria</td>
<td>JQ905065</td>
<td>99.8</td>
</tr>
<tr>
<td>7</td>
<td>OW12</td>
<td>Sagittula stellata</td>
<td>α-Proteobacteria</td>
<td>JQ905066</td>
<td>98.2</td>
</tr>
<tr>
<td>8</td>
<td>OW16*</td>
<td>Nitratireductor kinnyeongensis</td>
<td>α-Proteobacteria</td>
<td>JQ905067</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>OW18</td>
<td>Bacillus aryabhattai</td>
<td>Bacilli</td>
<td>JQ905068</td>
<td>98.9</td>
</tr>
<tr>
<td>10</td>
<td>OS1B</td>
<td>Bacillus nealsonii</td>
<td>Bacilli</td>
<td>JQ905069</td>
<td>98.3</td>
</tr>
<tr>
<td>11</td>
<td>OS1C</td>
<td>Bacillus oceanisediminis</td>
<td>Bacilli</td>
<td>JQ905070</td>
<td>98.6</td>
</tr>
<tr>
<td>12</td>
<td>OS11</td>
<td>Bacillus tequilensis</td>
<td>Bacilli</td>
<td>JQ905071</td>
<td>97.8</td>
</tr>
<tr>
<td>13</td>
<td>OS12</td>
<td>Bacillus circulans</td>
<td>Bacilli</td>
<td>JQ905072</td>
<td>99.7</td>
</tr>
<tr>
<td>14</td>
<td>OS20</td>
<td>Bacillus tequilensis</td>
<td>Bacilli</td>
<td>JQ905073</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>OS22</td>
<td>Bacillus nealsonii</td>
<td>Bacilli</td>
<td>JQ905074</td>
<td>98.7</td>
</tr>
<tr>
<td>16</td>
<td>OS36B</td>
<td>Bacillus aryabhattai</td>
<td>Bacilli</td>
<td>JQ905075</td>
<td>99.3</td>
</tr>
<tr>
<td>17</td>
<td>OS38A*</td>
<td>Pseudomonas stutzeri</td>
<td>γ-Proteobacteria</td>
<td>JQ905076</td>
<td>98.2</td>
</tr>
<tr>
<td>18</td>
<td>OS42</td>
<td>Bacillus megaterium</td>
<td>Bacilli</td>
<td>JQ905077</td>
<td>97.7</td>
</tr>
<tr>
<td>19</td>
<td>OS43</td>
<td>Bacillus nealsonii</td>
<td>Bacilli</td>
<td>JQ905078</td>
<td>97.6</td>
</tr>
<tr>
<td>20</td>
<td>OS46R</td>
<td>Bacillus flexus</td>
<td>Bacilli</td>
<td>JQ905079</td>
<td>99.0</td>
</tr>
<tr>
<td>21</td>
<td>OS53</td>
<td>Bacillus vallismortis</td>
<td>Bacilli</td>
<td>JQ905080</td>
<td>100</td>
</tr>
<tr>
<td>22</td>
<td>OSS4A</td>
<td>Bacillus oceanisediminis</td>
<td>Bacilli</td>
<td>JQ905081</td>
<td>99.6</td>
</tr>
<tr>
<td>23</td>
<td>NOS3</td>
<td>Bacillus vallismortis</td>
<td>Bacilli</td>
<td>JQ905082</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>NOS6</td>
<td>Bacillus tequilensis</td>
<td>Bacilli</td>
<td>JQ905083</td>
<td>99.3</td>
</tr>
<tr>
<td>25</td>
<td>NOS14</td>
<td>Bacillus vallismortis</td>
<td>Bacilli</td>
<td>JQ905084</td>
<td>100</td>
</tr>
<tr>
<td>26</td>
<td>NOS48</td>
<td>Bacillus soli</td>
<td>Bacilli</td>
<td>JQ905085</td>
<td>97.9</td>
</tr>
<tr>
<td>27</td>
<td>ASW1*</td>
<td>Pseudoalteromonas marinighatinosa</td>
<td>γ-Proteobacteria</td>
<td>JQ905086</td>
<td>99.8</td>
</tr>
<tr>
<td>28</td>
<td>ASW2A</td>
<td>Idiomarina baltica</td>
<td>γ-Proteobacteria</td>
<td>JQ905087</td>
<td>99.5</td>
</tr>
<tr>
<td>29</td>
<td>ASW2C</td>
<td>Staphylococcus succinus</td>
<td>Bacilli</td>
<td>JQ905088</td>
<td>99.9</td>
</tr>
<tr>
<td>30</td>
<td>ASW4A</td>
<td>Pseudoalteromonas sp.</td>
<td>γ-Proteobacteria</td>
<td>JQ905089</td>
<td><strong>96.8</strong></td>
</tr>
<tr>
<td>31</td>
<td>ASW4C*</td>
<td>Rheinheimera aquimarina</td>
<td>γ-Proteobacteria</td>
<td>JQ905090</td>
<td>98.7</td>
</tr>
<tr>
<td>32</td>
<td>ASW16</td>
<td>Pseudoalteromonas lipolytica</td>
<td>γ-Proteobacteria</td>
<td>JQ905091</td>
<td>98.3</td>
</tr>
<tr>
<td>33</td>
<td>ASS1</td>
<td>Bacillus tequilensis</td>
<td>Bacilli</td>
<td>JQ905092</td>
<td>98.8</td>
</tr>
<tr>
<td>34</td>
<td>ASS2A</td>
<td>Uncultured Marinobacter</td>
<td>γ-Proteobacteria</td>
<td>JQ905093</td>
<td><strong>94.7</strong></td>
</tr>
<tr>
<td>35</td>
<td>ASS2B*</td>
<td>Marinobacter guineae</td>
<td>γ-Proteobacteria</td>
<td>JQ905094</td>
<td>97.9</td>
</tr>
<tr>
<td>36</td>
<td>ASS3B*</td>
<td>Halomonas axialensis</td>
<td>γ-Proteobacteria</td>
<td>JQ905095</td>
<td>98.7</td>
</tr>
<tr>
<td>37</td>
<td>ASS4B</td>
<td>Bacillus subtilis</td>
<td>Bacilli</td>
<td>JQ905096</td>
<td>99.8</td>
</tr>
<tr>
<td>38</td>
<td>ASS5A*</td>
<td>Pseudoalteromonas sp.</td>
<td>γ-Proteobacteria</td>
<td>JQ905097</td>
<td><strong>96.8</strong></td>
</tr>
<tr>
<td>39</td>
<td>ASS5B*</td>
<td>Pseudoalteromonas lipolytica</td>
<td>γ-Proteobacteria</td>
<td>JQ905098</td>
<td>98.1</td>
</tr>
</tbody>
</table>

* Siderophore producing bacteria in this study. Serial numbers 1–26 are Indian Ocean bacteria; 27–39 are Arabian Sea bacteria.
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Similar results were also obtained through γ-Proteobacteria *Marinobacter* and *Halomonas* by Edwards *et al.* (2003).

Among the three main classes from across the EIO and AS, samples *Bacillus* spp. being one of the key organisms in marine heterotrophic bacterial community. This is because of their diverse nature and widespread adaptability to the environmental conditions (Parvathi *et al.*, 2009). Most of the *Bacillus* spp. obtained here were from the sediment samples at deeper depth (approx 4700 m) and that could be known for its adaptation and survival through spore forming in geologic environments (Vreeland *et al.*, 2000). We have noticed an interesting thing that the bacteria belong to α-Proteobacteria obtained only from the EIO water samples (Table I and Fig. 2a) and *Pseudoalteromonas* sp. could retrieve only from AS samples. *Halomonas*, *Rheinheimera*, *Staphylococcus* were some of the other genera obtained from AS samples (Fig. 2b). *Marinobacter* spp were common in both the study area.

Among the 39 strains tested for siderophore production, 9 of them produced yellow/orange colour around the colony (Fig. 3). Maximum diameter of the zone was 3 cm produced by *Pseudoalteromonas* sp. (ASW1, ASS5A and ASS5B) (Table II). Interestingly all the three *Pseudoalteromonas* sp. were affiliated to different 16S rRNA gene sequences which is evidenced in phylogenetic tree. Among the 9 strains produced siderophores 8 of them belong to γ-Proteobacteria and the remaining one to α-Proteobacteria. Bacteria belong to γ-Proteobacteria like *Marinobacter* and *Halomonas* are known for its production of self-assembling amphiphilic siderophores (Martinez *et al.*, 2000). Our studies were also in supportive of the above by saying *Marinobacter*, *Halomonas*, *Pseudoalteromonas*, *Rheinheimera* and *Pseudomonas* could produce siderophores in a better way. Though the previous reports say the transport of iron through siderophores by *Bacillus* spp. (Dertz *et al.*, 2003; Zawadzka *et al.*, 2009) we did not see siderophore production from *Bacillus* spp. The reasons are not known.

Siderophore production tested among the 9 strains using various techniques expressed its maximum production from the ASS2b strain with 87.9%. The lowest production was noticed in ASW4c at the rate of 44.4% (Table II). While comparing the siderophore production rates, the AS strain has given much higher when compared to EIO. Quantitative estimation of siderophores have shown that 6th day old culture produces maximum amount of siderophores as compared to 12th day old culture. Further the samples which produced siderophores were scanned to get a rough idea of the compounds present over there. All the 9 strains scanned for siderophore production indicate 2 peaks, one at 306 and the other at 246 nm (Fig. 4) (UV-scan data are not presented beyond 400nm in Figure 4 since there is no peak after). These peaks indicate the compound Azurechelin (Sokol *et al.*, 1992). They say that 88% of siderophore production by *Pseudomonas cepacia* had absorbance maxima at 240 and 310 nm. Our reports also indicate the peak in a similar fashion, it could be interpreted that the EIO and AS samples may produced the same compound.

Most of the microbial siderophores are either hydroxamate or catechol or carboxylate types (Miethke and Marahiel, 2007). All the bacteria from the present study were showing the absorption maxima between 190 and 280 (Spectrophotometric Scan) which indicate the presence of carboxylate type. Interestingly, bacterial siderophore production studies conducted by Sullivan *et al.* (2012) also revealed that in general 90% of the bacterial isolates were able to produce carboxylates type siderophores.

**Table II**

| Test isolates | Isolation source | Siderophore production zone in CAS plates (cm) | % of siderophore production
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6th day</td>
</tr>
<tr>
<td>ASW1</td>
<td>AS</td>
<td>3</td>
<td>85.3</td>
</tr>
<tr>
<td>ASW4C</td>
<td>AS</td>
<td>1</td>
<td>44.4</td>
</tr>
<tr>
<td>ASS2B</td>
<td>AS</td>
<td>2</td>
<td>87.9</td>
</tr>
<tr>
<td>ASS3B</td>
<td>AS</td>
<td>1</td>
<td>87.5</td>
</tr>
<tr>
<td>ASS5A</td>
<td>AS</td>
<td>3</td>
<td>81.3</td>
</tr>
<tr>
<td>ASS5B</td>
<td>AS</td>
<td>3</td>
<td>78.3</td>
</tr>
<tr>
<td>OW10</td>
<td>EIO</td>
<td>1</td>
<td>78.6</td>
</tr>
<tr>
<td>OW16</td>
<td>EIO</td>
<td>2</td>
<td>59.1</td>
</tr>
<tr>
<td>OS38A</td>
<td>EIO</td>
<td>2</td>
<td>73.3</td>
</tr>
</tbody>
</table>

(iron bacteria). Rheinheimera, Staphylococcus were some of the other genera obtained from AS samples (Fig. 2b). *Marinobacter* spp were common in both the study area.
Importance of heterotrophic bacteria in the cycling of carbon and nutrients, including iron is not fully understood (Tortell et al., 1996). This is especially so from oligotrophic waters (Fukuda et al., 1998). In general, the dissolved Fe concentration in the surface mixed layer was lower due to biological removal and
excess concentration of Fe-binding organic ligands (Nakabayashi et al., 2001 and 2002). Studies by Price et al. (1994) and Cochlan (2001) in the eastern Equatorial Pacific Ocean, and Pakulski et al. (1996) in Gerlache Strait explained that, even small quantities of iron could increase the heterotrophic bacterial abundance.

Current study on the diversity of culturable iron bacteria from the EIO and AS samples showed that the bacteria retrieved from the selective medium did not indicate any typical iron bacteria like Thiobacillus. On the other hand the bacterial groups like Pseudoalteromonas, Halomonas, Idiomarina, Erythrobacter, and Nitratireductor were retrieved during this study. These kind of heterotrophic organisms were well reported earlier for iron oxidation. Distinct variation on the diversity of iron bacteria were noticed in the analyzed sites indicating Erythrobacter, Roseovarius, Sagittula and Nitratireductor from the oligotrophic waters of EIO and Pseudoalteromonas, Halomonas, Rheinheimera, Staphylococcus and Idiomarina in nutrient rich waters of AS. Very few isolates like Marinobacter and Bacillus were common in both the seas.

Iron can be used in two ways by the microorganisms, one as an electron acceptor which oxidize iron directly and the others which produce siderophores to solubilize the iron for its nutritional requirements. Since the isolates Marinobacter hydrocarbonoclasticus, Nitratireductor kimyeongensis and Pseudomonas stutzeri (EIO) and Pseudoalteromonas spp, Marinobacter guineae, Rheinheimera aquimarins and Halomonas axialensis (AS) exhibit to produce siderophores, we assume that these organisms meet their iron requirement through siderophore production. Though Bacillus spp reported for siderophore production earlier, our investigations did not come across Bacillus spp. for siderophore production in the study area and the reasons are yet to be studied. In general from the current study it may be interpreted that the bacterial species from the oligotrophic waters like EIO try to obtain their iron requirement by oxidation and the bacteria of AS through siderophore production. Further most of the iron solubilising marine bacteria expected to synthesize similar type of compounds in their siderophore. This work reported to bring few new species and genus in bacterial diversity on iron oxidation.

Acknowledgements

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Literature


Characterization of *Klebsiella pneumoniae* Strains Isolated from Urinary Tract Infections: Detection of ESBL Characteristics, Antibiotic Susceptibility and RAPD Genotyping

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2 Faculty of Dentistry Research Center, Selcuk University, Konya, Turkey
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Abstract

In this study, a hundred *Klebsiella pneumoniae* strains isolated from urinary tract infections were evaluated in terms of genotyping, susceptibility to certain antibiotics and detection of extended spectrum of beta lactamase (ESBL) production. The random amplified polymorphic DNA (RAPD-PCR) method was used to identify the genetic differentiation of *K. pneumoniae* isolates. A total of 26 different DNA bands ranging between 334 bp and 28033 bp were detected among the strains. It was found that 100 *K. pneumoniae* strains revealed 11 different RAPD profiles. Antibiotic susceptibility tests were conducted using a disc diffusion method against 16 antibiotics. Fifty-five different resistance profiles were determined among the strains. ESBL-productions of the strains were determined by the double disc synergy test (DDST) and ESBL E-test methods. ESBL production rates among the strains were found to be 55% by E-test method and 45% by DDST method. While ESBL-producing *K. pneumoniae* strains showed the greatest resistance to penicillin G (100%), followed by piperacillin (92.7%) and erythromycin (85.4%), the resistance rates of non ESBL-producing strains to those antibiotics were determined as 97.8%, 88.8% and 88.8%, respectively. Both groups of strains showed the highest sensitivity to meropenem. Based on the results obtained from the study, it was concluded that the detection of ESBL-producing strains by the E-test method was more sensitive than by the DDST method. Phenotypic and genotypic identification methods should be used together to detect ESBL presence. The RAPD-PCR method alone will not be adequate in the genotyping of the strains and alternative DNA-based methods should be used.

**Key words:** *Klebsiella pneumoniae*, antibiotic resistance, ESBL, E-test method, RAPD typing

Introduction

ESBL-producing bacteria are increasingly causing urinary tract infections (UTI) both in hospitalized and outpatients. The increase of drug resistance among these organisms has made the therapy of UTI difficult and has led to greater use of expensive broad spectrum antibiotics such as third generation cephalosporins. Detection of ESBLs using conventional antimicrobial susceptibility methods and a delay in the detection and reporting of ESBL production by gram-negative bacilli are associated with prolonged hospital stay and increased morbidity, mortality and health care costs. (Mehrgan and Rahbar, 2008). The detection of ESBL production is important. ESBL positive strains are associated with increased mortality when compared to ESBL negative strains (Kim and Pai, 2002).

*Klebsiella* species, particularly *Klebsiella pneumoniae*, are important opportunistic nosocomial pathogens causing a variety of infections including urinary tract infections, pneumonia, septicemia, wound infections and infections in intensive care units. It has been estimated that *Klebsiella* spp cause 5–7% of the total bacterial nosocomial infections (Podschun and Ullmann, 1998). Since 1983, multi-resistant *K. pneumoniae* has been increasingly recognized internationally as a cause of hospital-acquired infections. Because of the acquisition of plasmids which code for the production of extended-spectrum β-lactamases (ESBL), ESBL-producing microorganisms are more resistant to extended-spectrum cephalosporins and aminoglycosides than non ESBL-producing microorganisms (Eisen *et al.*, 1995).

Most of the ESBLs in *E. coli* and *K. pneumoniae* are derived from TEM or SHV type β-lactamases by one or more amino acid substitutions that confer resistance to extended-spectrum cephalosporins. Recently more non-TEM and non-SHV derived ESBLs such as...
Experimental

Material and Methods

Sample collection and isolation of strains. A hundred *K. pneumoniae* strains isolated from urine samples belonging to patients with urinary tract infections were collected during May 2010–July 2011. The collected samples were inoculated in sterile Petri dishes containing ready prepared EMB agar and blood agar media and incubated at 35°C for 18–24 hours. The isolated colonies were subcultured and purified for characterization. The isolated bacteria were identified according to Bergey’s Manual of Determinative Bacteriology (Brenner, 1986) and confirmations of the strains were carried out using the API-20E (BioMérieux) test system.

Identification of *K. pneumoniae* isolates by API 20E test. The API 20E test was performed in accordance with the manufacturer’s protocol (BioMérieux, Marcy l’Étoile, France). All cultures were transferred onto 5% sheep blood agar plates prior to the inoculation of the API 20E strips. A bacterial suspension approximating a 0.5 McFarland standard was used for inoculation. All strips were incubated at 35°C for 24 h. The addition of reagents and the interpretation of reactions were performed in accordance with the manufacturer’s directions. The 20 biochemical test reactions on the strip were converted into an octal profile number. Each profile number was then decoded using the Analytical Profile Index. Diagnosis of *Klebsiella* spp. was conducted by Apiweb™.

Antibiotic susceptibility test. The standard Kirby-Bauer disk diffusion method was used to determine the antibiotic sensitivity profiles of the *K. pneumoniae* isolates (CLSI, 2008) for 16 antimicrobial agents [meropenem (10 µg), aztreonam (30 µg), erythromycin (10 µg), ciprofloxacin (5 µg), piperacillin (30 µg), penicillin G (10 µg), norfloxacin (10 µg), tetracycline (30 µg), netilmicin (30 µg), chloramphenicol (30 µg), amoxicillin/clavulanic acid (30 µg), cephalixin (30 µg), ceфикsin (30 µg), cefazidime (30 µg), ceftriaxone (30 µg) and cefotaxime (30 µg)]. A 12 cm Mueller-Hinton Agar (MHA) medium plate was swabbed with BHI (Brain Heart Infusion) broth inoculated with *K. pneumoniae* and incubated to a turbidity of 0.5 McFarland standards. Sixteen commercially prepared antimicrobial agent disks were placed on the inoculated plates. The plates were incubated at 35°C for 18 to 20 h. The diameters (in millimeters) of the clear zones of growth inhibition around the antimicrobial agent disks, including the 6 mm disk diameter, were measured using a ruler. Zone diameters were interpreted using guidelines from the CLSI (2008). *E. coli* ATCC 25922 was used for quality control.

All samples were screened for the production of an ESBL by the double disc synergy test (DDST) as described by Jalier et al. (1998) and the E-test (Biomerieux). The strains were pre-incubated in brain heart infusion broth (BHIB) at 37°C and the optimal density of 0.5 McFarland standards. This bacterial suspension was swabbed with sterile cotton on to a Mueller-Hinton agar medium. The antagonistic tests were conducted with antibiotic discs of amoxicillin/clavulanic acid (20/10 µg) and cefotaxime (30 µg), cefazidime (30 µg) and ceftriaxone (30 µg) were placed at a distance of about 4 cm apart from each other and incubated. After incubation a clear extension of the edge of the inhibition zone of any of the antibiotics towards the disk containing clavulanic acid-CA (10 µg) was interpreted as positive for ESBL production.

An E test ESBL strip (Biomerieux) is a plastic drug-impregnated strip, one end of which generates a stable concentration gradient of cefazidime (MIC test range, 0.25–16 mg/l) and the remaining end of which generates a gradient of cefazidime (MIC test range, 0.016–1 mg/l) plus 4 mg/l clavulanic acid. The E-test procedure, reading and interpretation were carried out according to the manufacturer’s instructions. Isolated colonies from an overnight agar plate were suspended in saline (0.85% NaCl) to achieve an inoculum equivalent to 0.5 McFarland standards. This suspension was swabbed on a Mueller-Hinton agar plate (Oxoid, UK) and allowed to dry completely. An ESBL E-test strip

as CTX-M related enzymes have been identified over an extremely wide geographical area (Li et al., 2003). These multi-resistant isolates produce the novel plasmid-mediated β-lactamases CTX-1, SHV-2 and SHV-3 which are able to hydrolyze oxyimino β-lactamases (Pitout et al., 2005).

Among all the genome fingerprinting PCR methods, random amplified polymorphic DNA (RAPD) is used for demonstrating differences between bacteria. RAPD can be used for the typing of organisms without previous knowledge of DNA sequences (Mohamudha et al., 2010, Dobara et al., 2010). The use of a single primer leads to the amplification of several DNA fragments randomly distributed throughout the genome. RAPD has received considerable attention in recent years as a molecular typing method due to its simplicity, sensitivity, flexibility and relatively low cost. The ability of RAPD to type a wide variety of bacteria strains in a short time suggests that it will be a useful molecular epidemiological tool (Dobara et al., 2010).

The aim of the present study was to investigate the presence and rates of ESBL and antibiotics susceptibilities and the genotypic identification of *Klebsiella pneumoniae* strains isolated from urinary tract infections in a hospital in Konya, Turkey.
was then applied to the agar surface with sterile forceps and the plate was incubated at 36°C for 18 h. The ESBL results were read either as MIC values or observation of ‘phantom’ zones or deformation of inhibition ellipses. A reduction of MIC by ≥3 two-fold dilutions in the presence of clavulanic acid is indicative of ESBL production. In addition, multiple antibiotic resistance (MAR) indexing of *K. pneumoniae* strains was determined according to Krumperman (1983).

**Extraction of genomic DNA.** DNA genomes were extracted from the bacterial isolates using a DNA extraction kit (Dr Zeydanlı, Life Sciences) as directed by the manufacturer and the supernatants were stored in a freezer at –20°C for PCR analysis.

**Random Amplified Polymorphic DNA (RAPD) Fingerprinting.** RAPD was carried out according to Williams *et al.* (1990) with some modification. PCR reactions were prepared in a total volume of 50 µl per tube, containing 5 µl (50 ng/µl) of chromosomal DNA, 0.7 µl (5U/µl) Taq DNA polymerase (Fermentas, Thermo Scientific), 3 µl MgCl₂ (25 mM), 5 µl taq buffer, 30.3 µl PCR buffer, 1 µl (25 pmol) primer (OPA-02). The following primer (OPA-02 5’ TGCCGAGCTG3’ ) was used in this study.

The amplifications were done in a thermal cycler (Eppendorf) programmed for the first cycles to denature for 1 min at 95°C. Then came a program of 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 36°C and a 2 min primer extension at 72°C followed by a final step of amplification of 7 min at 72°C.

**Gel preparation, sample loading and detection.** Amplification products were resolved by electrophoresis on 1.2% agarose gel that was prepared in 1X TBE (Tris/Borate/EDTA) buffer and mixed with 0.5 µg/ml of ethidium bromide. The agarose gel was transferred to an electrophoresis cell with 1X TBE buffer. 20 µl of each samples was mixed with 4 µl loading dye and loaded into the gel and 5 µl of DNA markers (Gene ruler 100 bp plus, Lambda DNA/Hind III) (Fermentas, Thermo Scientific) and Sigma Direct Load (Sigma Aldrich) were loaded into the well of the gel. 80 volts for 2 hours as 7.5 v/cm of the gel was applied. DNA bands were visualized using a 366 nm UV transilluminator and photographed with the UVP GelDoc It™ Imaging System.

**Results**

In this study it was found that 45 (45%) *K. pneumoniae* strains produced ESBL in the double discs synergy test and 55 (55%) strains produced ESBL in the E-test. Both methods demonstrated the presence of ESBL in 55 of the isolates. ESBL-producing *K. pneumoniae* strains displayed higher antibiotics resistance ratios than non ESBL-producing *K. pneumoniae* strains. Table I shows the antibiotic resistance and susceptibility ratios of ESBL-producing and non ESBL-producing *K. pneumoniae*.

The antibiotic susceptibility test by disc diffusion method found that ESBL-producing *K. pneumoniae*

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>non ESBL-producing <em>K. pneumoniae</em></th>
<th>ESBL-producing <em>K. pneumoniae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R* n (%)</td>
<td>I n (%)</td>
</tr>
<tr>
<td>Meropenem (MEM)</td>
<td>1 (2.2%)</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>Aztreonam (ATM)</td>
<td>7 (15.5%)</td>
<td>3 (6.6%)</td>
</tr>
<tr>
<td>Erythromycin (E)</td>
<td>40 (88.8%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>3 (6.6%)</td>
<td>4 (8.6%)</td>
</tr>
<tr>
<td>Penicillin G (P)</td>
<td>44 (97.8%)</td>
<td>–</td>
</tr>
<tr>
<td>Piperacilline (PRL)</td>
<td>40 (88.8%)</td>
<td>3 (6.6%)</td>
</tr>
<tr>
<td>Norfloxacin (NOR)</td>
<td>3 (6.6%)</td>
<td>(8.8%)</td>
</tr>
<tr>
<td>Tetracycline (TE)</td>
<td>12 (26.6%)</td>
<td>2 (4.6%)</td>
</tr>
<tr>
<td>Netilmicin (NET)</td>
<td>4 (8.6%)</td>
<td>–</td>
</tr>
<tr>
<td>Chloramphenicol (C)</td>
<td>5 (11.1%)</td>
<td>4 (8.8%)</td>
</tr>
<tr>
<td>Amoxicillin/Clavulanic acid (AMC)</td>
<td>12 (26.6%)</td>
<td>5 (11.1%)</td>
</tr>
<tr>
<td>Ceftazidime (CAZ)</td>
<td>6 (13.3%)</td>
<td>5 (11.1%)</td>
</tr>
<tr>
<td>Ceftriaxon (CRO)</td>
<td>5 (11.1%)</td>
<td>14 (31.1%)</td>
</tr>
<tr>
<td>Cefotaxime (CTX)</td>
<td>5 (11.1%)</td>
<td>4 (8.8%)</td>
</tr>
<tr>
<td>Cefoxilene (CL)</td>
<td>9 (20%)</td>
<td>11 (24.4%)</td>
</tr>
<tr>
<td>Cefotaxine (FOX)</td>
<td>8 (17.7%)</td>
<td>6 (13.3%)</td>
</tr>
</tbody>
</table>

* R: Resistant; I: Intermediate; S: Susceptible
strains showed the highest antibiotic resistance to penicillin in 55 strains (100%); piperacillin in 51 strains (92.7%); erythromycin in 47 strains (85.4%) and ceftriaxone in 28 (50.9%) strains respectively (Fig. 1). The strains showed the lowest antibiotic resistance to meropenem in 5 strains (9%); cefoxitin in 7 strains (12.7%); ciprofloxacin in 10 strains (18.1%) and norfloxacin in 12 strains (21.8%). ESBL-producing *K. pneumoniae* strains showed the highest antibiotic resistance to penicillin in 44 strains (97.8%); erythromycin and piperacillin in 40 strains (88.8%) and tetracycline and amoxicillin/clavulanic acid in 12 strains (26.6%) respectively. They showed the lowest antibiotic resistance to penicillin in one strain (2.2%); norfloxacin and ciprofloxacin in 3 strains (6.6%) and netilmicin in 4 strains (8.6%). It was found that non ESBL-producing strains showed high resistance to penicillin, erythromycin and piperacillin. ESBL-producing and non ESBL-producing *K. pneumoniae* strains showed the highest sensitivity to meropenem. While non ESBL-producing strains had high resistance to netilmicin and norfloxacin, ESBL-producing strains showed high sensitivity to cefoxitin and chloramphenicol.

Resistance profiles were formed based on the antibiotics that the strains were resistant to (Table II). According to this table, a total of 55 different resistance profiles were observed among 100 *K. pneumoniae* strains. The majority of strains (98%) were found to be resistant to two or more antibiotics. The most common resistance profile was identified in 23 strains and these
Table II
Antibiotic resistance patterns of *Klebsiella pneumoniae* isolates.

<table>
<thead>
<tr>
<th>Resistance profiles</th>
<th>Number of isolates</th>
<th>%</th>
<th>Strains</th>
<th>MAR index</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E</strong></td>
<td>1</td>
<td>1</td>
<td>51</td>
<td>0.0625</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>1</td>
<td>1</td>
<td>87</td>
<td>0.0625</td>
</tr>
<tr>
<td><strong>P, PRL</strong></td>
<td>3</td>
<td>3</td>
<td>8, 13, 35</td>
<td>0.125</td>
</tr>
<tr>
<td><strong>E, PRL</strong></td>
<td>4</td>
<td>4</td>
<td>14, 36, 48, 99</td>
<td>0.125</td>
</tr>
<tr>
<td><strong>E, P</strong></td>
<td>1</td>
<td>1</td>
<td>55</td>
<td>0.125</td>
</tr>
<tr>
<td><strong>E, P, PRL</strong></td>
<td>23</td>
<td>23</td>
<td>7, 9, 12, 21, 30, 31, 38, 37, 42, 47, 53, 54, 56, 58, 64, 67, 75, 78, 80, 82, 89, 96, 100</td>
<td>0.187</td>
</tr>
<tr>
<td><strong>P, PRL, CAZ</strong></td>
<td>1</td>
<td>1</td>
<td>20</td>
<td>0.187</td>
</tr>
<tr>
<td><strong>E, P, TE</strong></td>
<td>1</td>
<td>1</td>
<td>85</td>
<td>0.187</td>
</tr>
<tr>
<td>ATM, CAZ, CRO, FOX</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>0.25</td>
</tr>
<tr>
<td>ATM, E, P, PRL</td>
<td>3</td>
<td>3</td>
<td>59, 84, 97</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>E, CIP, P, PRL</strong></td>
<td>1</td>
<td>1</td>
<td>40</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>E, P, PRL, TE</strong></td>
<td>3</td>
<td>3</td>
<td>1, 23, 63</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>E, P, PRL, C</strong></td>
<td>3</td>
<td>3</td>
<td>15, 16, 86</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>E, P, PRL, AMC</strong></td>
<td>3</td>
<td>3</td>
<td>18, 19, 95</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>E, P, PRL, CL</strong></td>
<td>1</td>
<td>1</td>
<td>26</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>E, P, AMC, CL</strong></td>
<td>1</td>
<td>1</td>
<td>32</td>
<td>0.25</td>
</tr>
<tr>
<td>CIP, P, PRL, NOR</td>
<td>1</td>
<td>1</td>
<td>41</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>P, PRL, TE, FOX</strong></td>
<td>2</td>
<td>2</td>
<td>71, 72</td>
<td>0.25</td>
</tr>
<tr>
<td>ATM, E, CIP, P, PRL</td>
<td>1</td>
<td>1</td>
<td>43</td>
<td>0.313</td>
</tr>
<tr>
<td><strong>E, P, PRL, TE, C</strong></td>
<td>1</td>
<td>1</td>
<td>50</td>
<td>0.313</td>
</tr>
<tr>
<td><strong>E, P, PRL, TE, AMC</strong></td>
<td>3</td>
<td>3</td>
<td>22, 49, 74</td>
<td>0.313</td>
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<tr>
<td><strong>E, P, PRL, TE, CL</strong></td>
<td>1</td>
<td>1</td>
<td>39</td>
<td>0.313</td>
</tr>
<tr>
<td><strong>E, P, AMC, CL, FOX</strong></td>
<td>1</td>
<td>1</td>
<td>46</td>
<td>0.313</td>
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<tr>
<td>ATM, E, P, PRL, NET, CAZ, CRO</td>
<td>1</td>
<td>1</td>
<td>52</td>
<td>0.375</td>
</tr>
<tr>
<td><strong>E, P, PRL, TE, C, AMMC</strong></td>
<td>2</td>
<td>2</td>
<td>10, 29</td>
<td>0.375</td>
</tr>
<tr>
<td><strong>P, PRL, TE, AMC, CAZ, CL</strong></td>
<td>1</td>
<td>1</td>
<td>17</td>
<td>0.375</td>
</tr>
<tr>
<td>ATM, E, P, PRL, CAZ, CRO, CL</td>
<td>1</td>
<td>1</td>
<td>11</td>
<td>0.438</td>
</tr>
<tr>
<td>ATM, E, P, PRL, CAZ, CL, FOX</td>
<td>1</td>
<td>1</td>
<td>93</td>
<td>0.438</td>
</tr>
<tr>
<td>ATM, E, P, PRL, CRO, CTX, CL</td>
<td>1</td>
<td>1</td>
<td>73</td>
<td>0.438</td>
</tr>
<tr>
<td>ATM, E, P, PRL, CAZ, CRO, CTX, CL</td>
<td>1</td>
<td>1</td>
<td>45</td>
<td>0.438</td>
</tr>
<tr>
<td><strong>E, P, PRL, C, CRO, CTX, CL</strong></td>
<td>1</td>
<td>1</td>
<td>88</td>
<td>0.438</td>
</tr>
<tr>
<td><strong>E, P, PRL, TE, NET, C, AMC</strong></td>
<td>1</td>
<td>1</td>
<td>90</td>
<td>0.438</td>
</tr>
<tr>
<td>ATM, E, P, PRL, NOR, CRO, CTX, CL</td>
<td>1</td>
<td>1</td>
<td>62</td>
<td>0.50</td>
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<tr>
<td>ATM, E, P, PRL, CAZ, CRO, CTX, CL</td>
<td>1</td>
<td>1</td>
<td>81</td>
<td>0.50</td>
</tr>
<tr>
<td>ATM, E, P, PRL, AMC, CAZ, CRO, CTX, CL</td>
<td>1</td>
<td>1</td>
<td>68</td>
<td>0.50</td>
</tr>
<tr>
<td>ATM, P, PRL, NET, CAZ, CRO, CTX, CL</td>
<td>1</td>
<td>1</td>
<td>44</td>
<td>0.50</td>
</tr>
<tr>
<td>ATM, TE, NET, CAZ, CRO, CTX, CL, FOX</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>E, CIP, P, PRL, NOR, TE, C, AMC</strong></td>
<td>1</td>
<td>1</td>
<td>92</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>E, P, PRL, AMC, CRO, CTX, CL, FOX</strong></td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>0.50</td>
</tr>
<tr>
<td>ATM, E, P, PRL, NET, CAZ, CRO, CTX, CL</td>
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<td>2</td>
<td>3, 70</td>
<td>0.563</td>
</tr>
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<td>1</td>
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<td>0.563</td>
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<tr>
<td><strong>E, P, PRL, TE, NET, C, CRO, CTX, CL</strong></td>
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<td>1</td>
<td>34</td>
<td>0.563</td>
</tr>
<tr>
<td><strong>E, P, PRL, AMC, CAZ, CRO, CTX, CL, FOX</strong></td>
<td>1</td>
<td>1</td>
<td>65</td>
<td>0.563</td>
</tr>
<tr>
<td>ATM, E, CIP, P, PRL, NOR, CAZ, CRO, CTX, CL</td>
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<td>2</td>
<td>4, 83</td>
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<tr>
<td>ATM, E, P, PRL, TE, C, CAZ, CRO, CTX, CL</td>
<td>2</td>
<td>2</td>
<td>60, 61</td>
<td>0.625</td>
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strains showed resistance to erythromycin, penicillin and piperacillin. This profile had a 23% rate among the strains. We determined 4 strains that developed resistance to all 16 antibiotics and only showed resistance to erythromycin and piperacillin. The majority of observed resistance profiles are observed to consist of one strain. Among all, 66, 76, 79 and 94 numbered strains showed the highest MAR index values. In 65 strains (65%) showing resistance to at least four antibiotics, the MAR index was greater than 0.2. According to Krumperman (1983), it is claimed that strains which have an index higher than 0.2 come from a location in which antibiotics are intensively used.

In the present study genotypic analyses were performed on *Klebsiella pneumoniae* strains using the RAPD-PCR method (Fig. 2). In 44 of 100 strains, we determined 11 different DNA profiles, with minimum1, maximum 9 DNA bands. A total of 26 different DNA bands ranging between 334 bp and 28033 bp were determined (Fig. 2). Among the strains in which a DNA band was identified, 9 DNA bands were found in 1; 3 DNA bands were found in 2; 2 DNA bands were found in 4 and 1 DNA band was found in 37 strains (Table III).

A total of 9 DNA bands were found in *K. pneumoniae* strain no 3. It was found that the size of this band varied between 334–2056 bp. 3 DNA bands were observed in each 13 (590–2583 bp) and 92 numbered strain (1604–1618 bp). Furthermore, 2 DNA bands with a size of 422–2648 bp were found in strains no 4, 11, 26 and 27. The most common profile among the strains was profile no 9 with a size of 28033 bp. The prevalence of this profile among strains was found to be 84.09% (Table III).

### Discussion

The identification of ESBL presence in microorganisms causing hospital and community-acquired infections is of great importance as antibiotic treatment

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**Table II continued.**

<table>
<thead>
<tr>
<th>Resistance profiles</th>
<th>Number of isolates</th>
<th>%</th>
<th>Strains</th>
<th>MAR index</th>
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<tbody>
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<td>ATM, E, P, PRL, TE, NET, AMC, CAZ, CRO, CTX, CL</td>
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<td>0.686</td>
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<td>1</td>
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<td>0.75</td>
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<td>1</td>
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<td>0.75</td>
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<td>1</td>
<td>57</td>
<td>0.75</td>
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<td>1</td>
<td>28</td>
<td>0.75</td>
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<tr>
<td>MEM, ATM, E, CIP, P, PRL, NOR, NET, AMC, CAZ, CRO, CTX, CL</td>
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<td>1</td>
<td>27</td>
<td>0.813</td>
</tr>
<tr>
<td>ATM, E, CIP, P, PRL, NOR, TE, NET, AMC, CAZ, CRO, CTX, CL</td>
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<td>1</td>
<td>98</td>
<td>0.813</td>
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**Table III**

<table>
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<th>DNA band sizes (bp)</th>
<th>K. pneumoniae strains</th>
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<td>2056, 1806, 1491, 1108, 918, 787, 722, 565, 334</td>
<td>3</td>
<td>P2</td>
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<td>2648, 600</td>
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<td>26</td>
<td>P7</td>
</tr>
<tr>
<td>899, 522</td>
<td>27</td>
<td>P8</td>
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<td>28033</td>
<td>28, 29, 30, 31, 32, 33, 35, 36, 37, 38, 39, 40, 41, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 79, 80, 81, 83, 85, 86, 89, 95, 96</td>
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<td>1618, 1608, 1604</td>
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<td>P10</td>
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<tr>
<td>1618</td>
<td>97</td>
<td>P11</td>
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</table>
will be determined accordingly, treatment costs will be reduced, and mortality and morbidity will decrease. Firstly the ESBL enzyme produced by the bacteria should be accurately identified (Isık et al., 2007). The CLSI has recommended scanning and phenotypic validation test based on the indication of beta-lactam/beta-lactamase inhibitor synergy for in vitro identification of ESBL-producing K. pneumoniae isolates. However, since DDST is still the most common, cheapest and easiest method for ESBL identification, it is used in various microbiology laboratories in Turkey and around the world. In the present study, 100 K. pneumoniae strains were analyzed in terms of ESBL using DDST and ESBL E-tests. Although the analysis which used the DDST found ESBL-producing strains at the 45% level, the E-test found ESBL-producing strains at the 55% level. In this case, the E-test method can be considered as more reliable than DDST in the identification of ESBL-producing strains. In a similar study, Abacioglu et al. (1995) identified the ESBL-producing K. pneumoniae strain rate at 50% using the DDST method and as 62.5% using the E-test method. Using the E-test method, Yucesoy et al. (1996) identified 57.1% ESBL in K. pneumoniae isolates, which are hospital infection pathogens. Isık et al. (2007), found a 63.7% ESBL production rate in K. pneumoniae strains using the DDST method and a 59.8% ESBL production rate using the E-test. The findings of our study are consistent with the findings of previous studies. Using the DDST method, Steward et al. (2001) identified ESBL production level as 84% in K. pneumoniae strains. Vecauteren et al. (1997) identified ESBL-producing strains at an 81% level using the E-test method and at 97% using the DDST method. Cormican et al. (1996) carried out a study on 82 clinical isolates and reported that the E-test was 100% sensitive. In the same study, the double disc synergy test had a sensitivity of 87%. As indicated in previous studies, the E-test method was found to be more sensitive than DDST in identifying ESBL presence. Similarly, Drieux et al. (2008) carried out a study on Klebsiella and E. coli strains and reported that the E-test method had 98.6% sensitivity, while the DDST method had 94.4% sensitivity. Similarly, in our study the E-test method was found to be more sensitive than DDST. ESBL ratios and antibiotic resistance status were compared to previous studies with reference to data obtained by us. G. Bindayna et al. (2009) identified ESBL presence at 24.3% in K. pneumoniae strains and reported that carbapenems were the most effective antibiotics. Jalalpour (2011) identified 36% ESBL presence in K. pneumoniae strains. Lin et al. (2012) identified ESBL presence at 14.9% in K. pneumoniae strains obtained from neonatal intensive care units and reported that imipenem was the most effective antibiotic against these strains. The ESBL ratio determined in our study is higher than the ratios of previous studies. However, our findings are consistent with previous studies in that the strains were most sensitive to carbapenems. In another study, Goyal et al. (2009), found 66.7% ESBL-producing K. pneumoniae strains and reported that all of the strains were sensitive to meropenem with the highest resistance to ciprofloxacin (93.7%). According to our findings, resistance to ciprofloxacin (18.1%) was much lower than the findings of previous researchers. Despite this, findings on meropenem sensitivity were consistent with our findings. Sensitivity to ciprofloxacin in our findings was found to be lower than the findings of Krawczyk et al. (2005) and higher than those of other researchers. Behrooozi et al. (2010) reported the incidence of ESBL-producing K. pneumoniae strains as 12% and that the strains were most sensitive to ofloxacin (28%), and most resistant (100%) ampicillin, ceftazidime and cefalotin (100%). Based on our findings, the identified resistance to ciprofloxacin (18.1%) and norfloxacin (21.8%) among quinolone group antibiotics were consistent with the findings of Behrooozi et al. (2010); however ESBL-producing strains were the most sensitive to (9%) meropenem. Ceftazidime resistance was found to be 45.4%. Ejaz et al. (2011) identified 71.7% ESBL prevalence in K. pneumoniae strains isolated from urinary tract infections. They determined the highest resistance to cefazidime (100%), cefotaxime (98.7%) and cefuroxime (98.1%) and the lowest resistance to meropenem (3.6%). In our study, resistance levels to cefazidime (45.4%), cefotaxime (47.2%) and the prevalence of ESBL-producing strains were found to be lower than the findings of Ejaz et al. (2011).

55 different resistance types were determined among K. pneumoniae strains. Comparison of the resistance profile with the profiles obtained from RAPD study showed that there was great phenotypic variety between the strains. However, the strains which were different in phenotype were divided into 11 different genotypes identified by RAPD. Analysis of the distribution of profiles with the same band size according to strains showed that these strains displayed multiple antibiotic resistances. In other words, the strains showing the same genotype were found to be phenotypically different. It was observed that 34 strains that had a DNA band with a size of 28033 bp were dominant among the 44 strains showing the band profile (Table III). The different phenotypes of these strains indicate that they come from regions where antibiotics are intensely used and thus they phenotypically differed by acquiring resistance to these antibiotics (Krumperman, 1983). Eisen et al. (1995) found that a single epidemic strain type in K. pneumoniae strains determined by RAPD-DNA, plasmid profile and phenotypic analyses was prevalent among the patients. Lopes et al. (2005) identified 26 RAPD genotypes among 30 K. pneumoniae
strains. In addition, the researchers identified 3 different antibiotic resistance profiles among these strains. The typing percentage of the researchers was higher than in our findings. Despite this, 55 different resistance profiles identified in our study are much higher than the data reported by Lopes et al. (2005). 34 (77.2%) of 44 strains typed by RAPD had one DNA band. In this case, it can be thought that K. pneumoniae strains with a 28033 bp DNA band can come from a dominant epidemic origin. Dobara et al. (2010) identified 5 different RAPD genotypes and 3 different antibiotic resistant profiles among K. pneumoniae strains. The genotype and phenotype percentages identified by the researchers were found to be lower than in our findings. Peng et al. (2002) found that 20 ESBL-producing K. pneumoniae strains formed 11 different types by RAPD method. In a similar study, Sharma et al. (2007) identified 23 different DNA profiles in 40 ESBL-producing K. pneumoniae strains using the RAPD method and found that DNA patterns at 300 bp size were common. The typing percentages determined by these researchers are consistent with our findings.

In conclusion, antimicrobial resistance is a rapidly changing challenge and an increase in antibiotic resistance levels of the bacteria has been a global problem. One of the most important reasons for this is indiscriminate use of antibiotics. As antibiotic use increases, bacteria develop resistance mechanisms and it becomes difficult to treat their infections. This increases treatment costs and extends treatment time. A detailed and accurate identification of the bacteria that cause infections helps clinicians. Phenotypic and genotypic identification methods should be used; comparative studies using more than one method should be conducted and more than one method should be used to identify ESBL presence. There are some advantages of the RAPD technique such as it requires no DNA probes or sequence information for the specific primers, and that it involves no blotting or hybridization steps, hence, it is quick, simple and efficient, and it requires only small amounts of DNA. Despite all these advantages, there are some disadvantages of this method. Nearly all RAPD markers are dominant, i.e. it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). Co-dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely. PCR is an enzymatic reaction, therefore, the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome. Thus, the RAPD technique is notoriously laboratory-dependent and needs carefully developed laboratory protocols to be reproducible. Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product. Thus, the RAPD results can be difficult to interpret. Another disadvantage is the lack of prior knowledge about the identity of the amplification products. Because of these reasons, the RAPD-PCR method alone will not be adequate for the genotyping of the strains. Our results should be developed by more sensitive DNA studies such as pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) and nucleotide sequence analysis.

ESBL production of K. pneumoniae strains that cause urinary system infections should be followed via genotypic and phenotypic methods; their types should be identified and periodical identification and surveillance studies should be carried out on antibiotic resistance levels.

Acknowledgement
We would like to thank Selcuk University Scientific Research Projects Coordinating Office (BAP) for supporting this project financially (Project No: 10401050).

Literature


Screening, Characterization and Biofilm Formation of Nickel Resistant Bacteria Isolated from Indigenous Environment

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A b s t r a c t

Nickel resistant bacteria (ZB, ZC, ZD, ZL, ZK and S1X) were isolated from industrial effluents and corroded iron pieces from indigenous environment of Punjab, Pakistan. These six strains could tolerate nickel at different levels with ZB, ZC, ZD, ZL, ZK, and S1X having 233, 225, 267, 233, 228 and 296 mM minimum inhibitory concentration (MIC) of nickel ions, respectively. These bacteria were sensitive to Cu^{2+}, Cr^{3+}, Co^{2+}, and Al^{3+} as they did not grow even in the presence of 1 mM concentration of all these ions in minimal medium, whereas all of them were resistant to Fe^{3+} up to 1.3 mM in minimal medium. The best appropriate temperature for nickel resistant bacteria was 37°C and all of them showed maximum growth at pH 8. These bacteria were characterized morphologically and biochemically. Biofilm forming ability of the bacteria was checked with and without nickel stress and it was found that strains ZK and S1X were able to form a compact biofilm even under nickel stress. The sequencing of 16S rRNA-encoding genes from these nickel resistant bacteria showed that they belonged to four different genera namely, Klebsiella, Pseudomonas, Bacillus and Cronobacter.

K e y w o r d s: Nickel resistant bacteria, minimal medium, minimum inhibitory concentration (MIC), biofilm

Introduction

During last few decades, increased industrialization has resulted in environmental contamination with various pollutants, among those heavy metals are of serious concern because food chains can accumulate these heavy metals, causing serious hazards to the environment (Chen et al., 2008; Durve et al., 2012; Wani and Khan, 2013). Nickel is being widely used in various industries such as leather tanning, electroplating, pulp processing, steel manufacturing and wood preservation and is discharged into wastewater and surrounding environment by these industries. This is of key concern because of non-degradable nature of nickel (Congeevaram et al., 2007; Karakagh et al., 2012). Nickel is typically found in Ni (0) or Ni (II) state due to the stability of these species in water (Niemiinen et al., 2007). Nickel is an essential compound for bacterial metabolism (Hausinger, 1987) and is used as a co-factor by several well-characterized microbial enzymes like urease, hydrogenase, Ni-superoxide dismutase, carbon monoxide dehydrogenase, acetyl CoA synthase/decarbonylase, and methyl coenzyme M reductase, as well as some forms of glyoxalase (Mulrooney and Hausinger, 2006; Ragsdale, 2009; Kaluarachchi et al., 2010; Li and Zamble, 2010), but at higher concentrations nickel becomes toxic (Nies, 1992). The bacterial strain which can resist Ni (II) concentration greater than 99.8 mg/l may be considered as nickel resistant bacterial strain (Duxbury, 1981). Bacterial resistance to nickel is dependent upon a specific efflux system which is an operon-encoded and energy-dependent system that pumps excess of Ni^{2+} out of the cell and thus lowers the intracellular Ni^{2+} concentration (Park et al., 2003; Mulrooney and Hausinger, 2006). The presence of nickel in the surrounding medium induces the expression of nickel resistant determinant in bacterial strains (Zhu et al., 2011).

Interestingly, biofilm formation in many bacterial species is motivated by some stresses such as elevated metal concentration or some non-optimal growth conditions in the immediate environment of bacterial cells (Castonguay et al., 2006; Harrison et al., 2007). A biofilm is an aggregation of microbial cells which can be established on different surfaces. Biofilm is encapsulated by a self-produced matrix of extracellular polymeric substances (EPS), which is mainly composed of polysaccharides, proteins, nucleic acids, and lipids (Flemming and Wingender, 2010; Abee et al., 2011). Studies have shown that bacterial cells in biofilms are more resistant to the detrimental effects of heavy
metals than planktonic cells as they show better survival than free floating bacteria in the metal contaminated environment (Booth et al., 2011; Ansari et al., 2012). The present study was aimed at the isolation, characterization and identification of nickel resistant bacteria, to find out minimum inhibitory concentration (MIC) of nickel metal and to check the biofilm formation of these isolated bacteria under nickel stress.

**Experimental**

**Materials and Methods**

**Sample Collection.** Wastewater samples (from Kot-Lakhpat Industrial Estate Lahore, Pakistan) and corroded iron pieces (from old iron market Lahore, Pakistan) were collected in screw capped sterilized bottles and plastic bags respectively. Some physicochemical parameters like pH and temperature of wastewater were measured at the site of collection.

**Isolation of Nickel Resistant Bacteria.** For the isolation of nickel resistant bacteria, 50 µl of wastewater and 50 mg of scratched corrosion product from corroded iron pieces were separately spread and sprinkled on nutrient agar (Cappuccino and Sherman, 2007) plates supplemented with 1 mM of nickel chloride (NiCl₂·6H₂O). The plates were incubated at 37°C for 24 hours. The bacterial colonies were selected and purified on nickel chloride (1 mM) supplemented nutrient agar plates. After purification, the selected bacteria were shifted to slightly modified minimal agar medium as described by Schmidt et al., 2007. Bacterial growth was checked with (1 mM) and without nickel metal. The modified minimal agar medium contained 1 g (NH₄)₂SO₄, 0.5 g K₂HPO₄, 0.2 g MgSO₄·7 H₂O, 0.01 g FeSO₄·7 H₂O, 10 g glucose, 15 g agar and 1 l distilled water. The plates were again incubated at 37°C for 24 hours.

**Characterization of nickel resistant bacteria.** Nickel resistant bacterial isolates were characterized morphologically and biochemically (Cappuccino and Sherman, 2007). Two parameters i.e., pH and temperature were selected to check optimum growth of bacterial isolates. For the determination of optimum temperature, three sets of test tubes with minimal broth medium were prepared and inoculated with overnight culture of each bacterium. The three sets were incubated overnight at 28, 37, and 45°C respectively and absorbance of cultures was measured at 600 nm using IRMECO uv-vis spectrophotometer. For the determination of optimum pH, four sets of test tubes with minimal broth medium were prepared and their pH was adjusted at 5, 6, 7, and 8 then autoclaved. These tubes were then inoculated with overnight culture of each strain. After overnight incubation, absorbance of cultures was measured at 600 nm using IRMECO uv-vis spectrophotometer.

**Determination of minimum inhibitory concentration (MIC) of nickel for the bacterial isolates.** Minimum inhibitory concentration (MIC) against nickel was determined by broth dilution method. Stock solution (1.26 M) of NiCl₂·6H₂O was prepared in sterile distilled water. Sterile minimal broth medium with varying concentrations (0 mM to 300 mM) of nickel metal was prepared in test tubes (5 ml broth per tube). Overnight bacterial cultures were then diluted to reach a final optical density of 0.3 at 600 nm (OD₆₀₀ = 0.3) for all the bacterial isolates, 50 µl of bacterial inoculum per tube was added to each set of tubes designated for respective bacteria. Ion supplemented minimal broth medium was used as negative control for each concentration of nickel. These tubes were then incubated for 24 hours at 37°C at 100 rpm. The MIC was defined as the lowest concentration of nickel metal at which the bacteria do not show visible growth (Randrianarivelo et al., 2009). The experiment was performed twice in duplicates and MIC is presented as mean values of the experimental results.

**Resistance to other heavy metal ions.** The resistance of these isolated bacteria against other heavy metals was checked in minimal agar medium. The other heavy metals used were as follows: Al₂(SO₄)₃·18H₂O, CuSO₄·5H₂O, FeCl₃·6H₂O, CoCl₂·6H₂O and K₂Cr₂O₇. The plates were incubated at 37°C and growth was observed till 48 hours.

**Determination of biofilm formation of bacterial cells.** A qualitative assay for biofilm formation of nickel resistant bacteria was performed in glass test tubes. Bacterial cultures were grown in minimal medium with and without nickel metal (170 mM) stress for 24 hours without agitation. After 24 hours, the liquid medium was removed, and the bacterial biofilm was visualized by following Qurashi and Sabri 2012.

**Determination of effect of nickel metal (170 mM) on planktonic, loosely attached and tightly bound cells/biofilm growth.** Biofilm formation of nickel resistant bacteria was quantified in terms of planktonic, loosely attached and tightly bound cells in borosilicate tubes with and without nickel added. Overnight bacterial cultures (in minimal broth medium) were standardized (OD₆₀₀ = 0.1) and 100 µl standardized cultures were inoculated into 5 ml of minimal broth medium. Tubes were incubated at 37°C for 72, 120, and 168 hours under static conditions. Two sets of tubes were used for each bacterial isolate, one set with nickel metal stress (170 mM) and one set as control without nickel metal stress. After incubation, bacterial cultures were processed as previously described by Liaqat et al., 2009. The experiment was performed twice in duplicates.

**Identification of bacterial isolates.** To identify the taxonomic position, the isolated bacteria were sent to Macrogen Inc. Seoul South Korea for 16S rRNA gene sequencing. Obtained sequences were analyzed using...
**Biofilm formation of nickel resistant bacteria**

Finch TV (Geospiza, Inc. Seattle, WA) software and compared with the known sequences in the GenBank database through the National Center for Biotechnology Information (NCBI) to identify the most similar sequence alignment. These sequences of nickel resistant bacteria were then deposited in GenBank in order to get the accession numbers.

**Statistical analysis.** The results obtained in the quantification of biofilm in terms of planktonic, loosely attached and tightly bound cells were statistically analyzed using two-way ANOVA.

**Results**

**Physicochemical characteristics of wastewater**

The pH of different wastewater samples ranged from 8 to 8.5 and temperature ranged from 36 to 39°C (Table I).

**Nickel resistant bacteria**

A total of 26 bacterial isolates were selected from different samples on nickel chloride supplemented (1 mM) nutrient agar plates. Fourteen strains were selected from plates spread with wastewater samples and 12 strains were selected from plates sprinkled with corrosion products from corroded iron pieces. These 26 bacterial isolates were purified and seeded into the elevated level of nickel metal in minimal medium. Total of 6 bacterial isolates (ZB, ZC, ZD, ZL, ZK, and S1X) were selected based on their high resistance to nickel metal in minimal medium.

**Characterization of nickel resistant bacteria**

The six nickel resistant bacterial strains were characterized morphologically and biochemically. The results are depicted in Table II. The optimum temperature for growth of nickel resistant bacteria was found to be 37°C and all the bacterial isolates showed maximum growth at pH 8 (Figure 1A and 1B).

**Minimum Inhibitory Concentration (MIC) of nickel metal for the selected bacterial isolates**

Minimum inhibitory concentration (MIC) of nickel ions for these selected bacterial isolates was determined...
by broth dilution method and MIC values ranged from 225 mM to 296 mM. The bacterial strains ZB, ZC, ZD, ZL, ZK, and S1X showed minimum inhibitory concentration (MIC) of nickel at 233, 225, 267, 233, 228 and 296 mM respectively.

Resistance to other heavy metal ions
These bacterial isolates (ZB, ZC, ZD, ZL, ZK, and S1X) were further tested for their resistance against various other heavy metals. All the isolates were sensitive to Cu$^{2+}$, Cr$^{3+}$, Co$^{2+}$, and Al$^{3+}$ as these bacteria did not show growth even at 1 mM concentration of all these metals in minimal medium, whereas all of these bacterial isolates were resistant to Fe$^{3+}$ up to 1.3 mM.

Biofilm formation of bacterial cells
Biofilm formed by the nickel resistant bacteria (ZB, ZC, ZD, ZL, ZK and S1X) was visualized as dark purple ring formed on the walls and base of the test tubes in a qualitative analysis.

Effect of nickel metal (170 mM) on planktonic, loosely attached and tightly bound cells/biofilm growth
The effect of nickel (170 mM) on the planktonic, loosely attached, and tightly bound cells of bacteria was studied. Strains ZB, ZC, ZK and S1X showed a decrease in planktonic and loosely attached cells under nickel stress as compared to control. An increase in tightly bound cells was observed for strains ZB, ZK and S1X from 72 to 168 hours in both control and Ni stressed medium. In case of strain ZC, an increase in amount of tightly bound cells was observed by 168 hours in control and by 120 hours under nickel stress. In strains ZD and ZL a decrease in number of planktonic cells was observed by 168 hours under Ni stress whereas number of loosely attached and tightly bound cells increased by 168 hours for ZD under nickel stress. In case of ZL, number of loosely attached cells increased under Ni stress whereas number of tightly bound cells/biofilm was the same in both control and under Ni stress (Figure 2 A-F).

Identification of bacterial isolates
The 16S rRNA gene sequencing revealed that ZB, ZC and ZL isolates showed sequence similarity (99%) to *Klebsiella pneumoniae* strain DSM 30104. ZD was 99% similar to *Cronobacter sakazakii* strain ATCC 29544. Whereas ZK and S1X showed sequence similarity (99%) to *Pseudomonas aeruginosa* strain DSM 50071 and *Bacillus subtilis* subsp. *subtilis* strain DSM 10 respectively. The nucleotide sequences coding for 16S rRNA genes of nickel resistant bacteria have been
submitted to NCBI GenBank database under accession numbers Bacillus subtilis strain S1X (KC243314), Klebsiella pneumoniae strain ZB (KC243315), Klebsiella pneumoniae strain ZC (KC243316), Cronobacter sakazakii strain ZD (KC243317), Pseudomonas aeruginosa strain ZK (KC243318) and Klebsiella pneumoniae strain ZL (KC243319).

**Statistical analysis**

Difference in planktonic, loosely attached and tightly bound cells, both in control and Ni stressed conditions at different incubation times, was analyzed using two-way ANOVA. Significant difference was observed (P < 0.05) in all three types of cells for all the bacterial strains (ZB, ZC, ZD, ZL, ZK and S1X) with
a few exceptions. Number of loosely attached cells for ZC and planktonic cells for ZD were not significantly different (P > 0.05) with respect to incubation times. For ZD and ZL significant difference was not found for tightly bound cells under control and stressed conditions (P > 0.05). In case of S1X, loosely attached cells were also not significantly different under control and Ni stress conditions (P > 0.05).

Discussion

The existence of heavy metals in the surroundings of microbes can affect their growth, morphology and biochemical activities (Gadd, 1992; Roane and Pepper, 1999). Microbes have evolved different types of resistance and tolerance mechanisms for their survival in metal contaminated environment. These mechanisms may include (i) specific efflux pumps to expel toxic metal out of the cell, (ii) aggregation of the toxic metals, (iii) reduction in the permeability of microbial cell membranes, (iv) enzymatic modification of toxic metals to a less toxic form (Nies, 1999; Bruins et al., 2000; Nies, 2006). Nickel resistant bacteria have been isolated from different Ni polluted environments as wastewater, mine refuse, industrial composts (e.g. metallurgical and batteries industries) and cooling water from the metal processing industry (Park et al., 2003). In this study, a total of six bacterial strains have been isolated showing minimum inhibitory concentrations (MIC) for Ni^{2+} in the range of 225 mM to 296 mM with the highest MIC value for isolate S1X (Bacillus sp.) and lowest value for isolate ZC (Klebsiella sp.). These higher values of MIC of nickel for the isolated bacteria may be attributed to the presence of a plasmid encoded inducible energy-dependent efflux pump (Liesegang et al., 1993). It has been reported that these nickel efflux pumps are best characterized in organisms exhibiting hyper-resistance to nickel metal, although nickel efflux is widely used by cells to protect against elevated concentrations of this metal, several other mechanisms are also utilized by microorganisms to combat the elevated nickel concentration (Macomber and Hausinger, 2011). All of these bacteria showed resistance upto1.3 mM for Fe^{3+} and were found sensitive to other heavy metals like Cu^{2+}, Co^{2+}, Al^{3+}, and Cr^{3+}. Nickel resistance has been reported in species of different bacterial genera such as Streptomyces (Amoroso et al., 2000; Karakagh et al., 2012), Pseudomonas sp. and Bacillus sp. (Pal et al., 2004; Karakagh et al., 2012), Pseudomonas putida MH1d, Enterobacter intermedius MH8b, Enterobacter intermedius AM15, Klebsiella pneumoniae AM12 (Markowicz et al., 2010), Methylobacterium oryzae strain CBMB20, Burkholderia sp. strain CBMB40 (Madhaiyan et al., 2007), Enterococcus sp. (De Niederhäusern et al., 2013), Micrococcus sp. (Congevaram et al., 2007). Geobacillus toebii subsp. decanicus and Geobacillus thermoleovorans subsp. stromboliensis (Özdemir et al., 2012). The survival of microbial cells under the influence of toxic compounds is a multifactorial phenomenon, which might be achieved by molecular mechanisms of resistance against these toxic compounds as well as by the development of biofilm on a substrate under stressed conditions (Harrison et al., 2007; Perrin et al., 2009).

In this present study the effect of nickel (170 mM) on the planktonic, loosely attached and tightly bound cells/biofilm has been studied for the bacterial strains ZB, ZC, ZD, ZL, ZK, and S1X. Generally, a trend for decrease in planktonic and loosely attached cells has been observed under nickel stress compared to control which might be the result of some toxic effects of nickel ions on bacterial cells. These toxic effects might involve (1) replacement of some essential metal of metalloproteins by nickel, (2) binding of nickel to catalytic residues of non-metal enzymes, (3) binding of nickel outside the catalytic site of an enzyme to inhibit allosterically and (4) oxidative stress caused by nickel that can affect proteins, DNA, or lipids (Macomber and Hausinger, 2011). In case of tightly bound cells or biofilm formation on the glass test tubes, the results vary among different bacterial strains. For strain ZB (Klebsiella sp.), a decrease in tightly bound cells has been observed under Ni stress, but with respect to time of incubation an increase in tightly bound cells has been observed from 72 hours to 168 hours in control as well as under nickel stress (Fig. 2A). In case of strain ZC (Klebsiella sp.) an increase in tightly bound cells has been observed after 120 hours in control medium which may be due to the depletion of nutrients in the medium which forced the bacterial cells to develop biofilm for their survival under this stress, whereas under Ni stress a decrease in tightly bound cells is observed after 120 hours which means that after 120 hours biofilm either stabilizes or bacterial cells start shedding from the surface (Fig. 2B) (Liaqat et al., 2009). For ZD (Cronobacter sp.) strain, tightly bound cells increase from 72 to 168 hours under control medium whereas, under Ni stress increase in tightly bound cells/biofilm has been observed after 120 hours. These observations may be attributed to the fact that after a certain time, depletion of nutrients and presence of metal stressor in the medium force the bacterial cells to change from free floating cells to biofilm mode which protects the cells under stressed conditions (Fig. 2C). For ZL (Klebsiella sp.) strain, tightly bound cells/biofilm is same both under control and Ni supplemented medium at all the incubation times (Fig. 2E). For ZK (Pseudomonas sp.) and S1X (Bacillus sp.) strains, an increase in tightly bound cells/biofilm has been observed under nickel stress compared to control and this increase has also been observed from 72 to
168 hours. Whereas in control medium biofilm formation has been found to be increasing after 120 hours which shows that bacterial cells have shifted from free floating form of life to biofilm mode for their survival under stressed conditions of nutrient deficiency and nickel concentration (Fig. 2D, 2F). It has been reported that sub inhibitory concentration of nickel metal urges Escherichia coli cells to develop biofilm for their survival under stressed conditions, rather than living as planktonic cells (Perrin et al., 2009). So from this study it can be suggested that nickel stress may force bacterial cells to alter their lifestyle from free floating cells to biofilms, to resist the toxic effects of nickel metal.

Remediation of heavy metals using microbial species is a well documented and efficient process. In this study heavy metal resistance pattern presented by studied bacteria was investigated and data show that bacteria were highly resistant to nickel and some of the strains show greater tendency to form biofilm as their survival strategy under this stressed condition. Biofilms are an appropriate source for the remediation of pollutants due to their high resistance and ability to immobilize the pollutants in the biofilm matrix. Hence, it can be suggested that these bacteria can be used as bioremediation tool for the treatment of industrial effluents.

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Literature


**Phylogenetic and Biochemical Characterization of a New Halo-Thermotolerant, Biofilm-Forming Bacillus from Saline Lake of Iran**

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**Abstract**

In this study, five halotolerant Bacillus isolates from Aran-Bidgol Saline Lake in Iran were identified from saline environments. Screening of the bacteria led to the identification of a unique halo-thermotolerant Bacillus. On the basis of genetic and phenotypic data, this isolate was closely related to Bacillus licheniformis. But isolated Bacillus can be distinguished from B. licheniformis by salt tolerance, 16S rDNA sequence and some different physicochemical properties. Thus, suggested that the isolate was not the known Bacillus. Optical density analysis indicated strong biofilm formation for this strain. Also this isolate exhibited average tolerance to 1–25 mM concentrations of zinc and was sensitive to all concentrations of nickel. In biosurfactant production assay, this Bacillus exhibited the high activity for semi-quantitative oil displacement test (3.14 ± 0.02 cm²) and evaluated positive for drop-collapse test and hemolytic activity. Moreover, amylase, protease and DNase enzymes produced in presence of 10–20% salt of medium. Therefore, identified Bacillus could supply potential microbial materials for bioremediation purposes and biotechnological applications.

**Key words:** biofilm formation, halo-thermotolerant Bacillus, phylogenetic analysis, Saline Lake

**Introduction**

The properties of saline and hypersaline habitats on earth are reflected in the great diversity within the microbial communities adapted to life under the prevailing conditions (Oren, 2002a). The aspects that attracted the attention of researchers were mainly those related to their physiological adaptation to highly saline concentrations and their ecology (Ghozlan et al., 2006; Ventosa et al., 1998a; 1998b). Recently, increasing interest, in microorganisms from hypersaline environments led to the discovery of several new bacterial species and genera (Yildiz et al., 2011). Besides their important role in the ecology of hypersaline environments, these prokaryotes, could be used in a multitude of potential applications in various fields of biotechnology (Coronado et al., 2000). They are a good quality source for compatible solutes that can be used as salt antagonists, stabilizers of biomolecules and whole cells, or stress-protective agents (Margesin and Schinner, 2001). Other useful bio-substances are exoenzymes, such as new isomerases and hydrolases that are active and stable at high salt concentrations. In addition, biopolymers such as biosurfactants and exopolysaccharides are of interest in enhanced oil recovery processes, degradation of industrial residues and toxic chemicals that can pollute hypersaline habitats has also been claimed (Coronado et al., 2000; Margesin and Schinner, 2001; Ventosa et al., 1998b).

On the other hand, in their natural environment, some halophilic bacteria occur in microbial aggregates as biofilm communities. It seems that the biofilm structure allows the attachment to various substrates and the
survival of cells by their interactions with ions such as heavy metals (Davey and Otoole, 2000; Mauger et al., 2010; Poli et al., 2010). Thus, detoxifying ability of these microorganisms can be manipulated for bioremediation of heavy metals in wastewater systems (Kamika and Momba, 2011). Therefore extreme environments can offer novel microbial biodiversity that produces varied and promising useful bio-substances for biotechnological applications (Llamas et al., 2010; Mata et al., 2006; Nichols et al., 2005). Aran-Bidgol Saline Lake in the central part of Iran is a hypersaline environment that similarly to other hypersaline ecosystems is subjected to drastic physicochemical conditions including high salinity, high radiation and strong changes in temperatures and dryness which make it an applicable study target for microbiologists.

In this study, we report the determination of phylogenetic properties, phenotypic features, physiologival and biochemical characteristics of a new halo-thermotolerant *Bacillus* from the Aran-Bidgol Saline Lake of Iran with a practical perspective on biotechnology. Also, important properties of this isolate such as biofilm formation ability, biosurfactant production, extracellular hydrolytic activates, nickel and zinc resistance were evaluated.

**Experimental**

**Material and Methods**

**Physicochemical analysis of the samples.** Samples for isolation of bacteria were collected 10 cm below the water surface of the lake in June 2011. Ion content of the water samples were measured according to standard methods (Lenore et al., 1989). Na⁺, K⁺ and Ca²⁺ were quantified by flame spectrophotometer (Genway, Uk), Mg²⁺ was quantified by atomic absorption spectrophotometer (Analytik Jena, Germany). PH and temperature were determined in situ.

**Enrichment, bacterial isolation and culture conditions.** Enrichment procedures were performed in medium including brine sample enriched with 2.5 g/l yeast extract (Difco) and 5 g/l tryptone (Difco). One hundred ml of each enriched medium was placed into 250 ml flasks. Cultures were incubated at 35°C and in an orbital shaker, at 150 rpm, during 3–7 days. After 4 days of incubation, the enrichment culture were spread on a saline nutrient agar plates, with a final concentration of 10% sea salt, containing (per liter): NaCl, 250 g; MgCl₂, 6H₂O, 13 g; MgSO₄, 7H₂O, 20 g; KCl, 4 g; CaCl₂, 2H₂O, 1 g; NaHCO₃, 0.2 g for moderately halophilic bacteria and 20% (w/v) for extremely halophilic microorganisms (Rohban et al., 2009), supplemented with 2.5% yeast extract and 5% tryptone, solidified with 10–12 g/l agar. Different colonies were picked and passaged several times to obtain pure cultures. Microbial cultures were stored at −80°C in the isolation medium supplemented with 10% glycerol.

**Morphological, physiological and biochemical tests.** All assay media for characteristics of the isolates were supplemented with 10% and 20% sea salt. Physiological and biochemical tests were performed as recommended by Smibert and Krieg (1994). Hydrolytic enzymes activities of isolates were screened qualitatively according to Rohban et al. (2009). Cultures were tested in triplicate and compared with negative and positive control. For assays of salt tolerance, cultures were incubated in nutrient broth containing 0, 2, 5, 7 and 10% (w/v) NaCl. Duplicate culture tubes containing 6 ml medium were inoculated with a loopful of 24 h culture grown in nutrient broth at 30°C. The inoculated tubes were incubated at 37°C and monitored for growth at 2 day.

**16S rDNA analysis.** The genomic DNA of isolates was extracted by QIAamp DNA Mini Kit (Qiagene, Germany) according to the manufacturer's instructions. The 16S rDNA gene was amplified by using primers FD1 (5′-CGAATTCGTCGACACAGATTGTAGCTCTGCGTCAAG-3′) and RP1 (5′-CCCGGGGATCCAAAGCTATACCTTGGTTACGACTT-3′) (Weisburg et al., 1991). PCR was performed in a thermal cycler (Bioneer, South Korea). The reaction mix included smarTaq DNA polymerase (Cinnagen, Iran) 0.25 μl, 0.5 μl of each primer, dNTP (10 mM) 0.5 μl, PCR buffer (10X) 2.5 μl, MgCl₂ (50 mM) 0.75 μl, template DNA 2 μl, and deH₂O 18 μl, in a final volume of 25 μl. The PCR amplification was performed using the following program: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 61°C for 30 s, and extension at 72°C for 2 min with final 10 min extension at 72°C. PCR product was analyzed on 1% agarose gel stained with ethidium bromide (0.5 μg/ml) and visualized under ultra violet transillumination (Syngene InGenius, US). PCR products were sequenced using an automated sequencer (ABI system, 3730XL) by Macrogen Company in Korea. The phylogenetic relationship of the isolates were determined by comparing with the related sequences in the GenBank database by advanced BLAST searches from National Center for Biotechnology Information. Among the isolated strains, SL1 isolate, was selected for further identification based on its high salt and thermo tolerance, hydrolytic activity, biochemical properties and 16S rDNA data

**Phylogenetic tree of SL1 isolate.** Phylogenetic tree analysis was performed by using the software package MEGA5 version (Tamura et al., 2011) after obtaining multiple alignments of data available from public databases using CLUSTAL W (Thompson et al., 1994). Pair
wise evolutionary distances were computed using the correction method and clustering was performed using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 2,000 replicates (Zharkikh and Li, 1995).

**Biofilm formation of SL1 isolate.** Quantitative analysis of biofilm production was performed as described by Seno et al. (2005), with some modifications. Briefly, the isolate was grown in tryptic soy broth (TSB) with 10% and 20% (w/v) sea salt. From each culture, 20 µl samples and 180 µl of TSB were dispensed in the wells of sterile 96-well flat-bottomed microtiter plate (BD Biosciences) and incubated at 35°C for 48 h. The control wells contained only TSB medium. After 48 h, wells were washed three times with distilled water, dried and fixed for 30 min at 80°C. Adhered cells were stained with 0.5% crystal violet solution for 30 min. The stain was washed out with distilled water. In order to quantify adhered cells, 220 µl of ethanol-acetic acid (95:5, vol/vol) was added to each well for 15 min. Optical density (OD) of eluted stain was measured at 590 nm by using a microtiter plate reader (Awareness technology INC, U.S). Each assay was performed triplicate. As a control uninoculated medium was used. The strain with $A_{590}<0.5$, and $A_{590}\geq0.5$ were defined as weak and strong biofilm formers, respectively.

**Biosurfactant production of SL1 isolate.** Examination of biosurfactant production and surface tension was performed by Drop-collapse, Oil displacement and hemolytic activity tests (Bodour et al., 2003; Morikawa et al., 1993). Briefly, the isolate was cultured in broth medium and incubated at 36°C for 3 days. Cell suspensions were centrifuged (10,000 g) and the cell-free supernatant was used for analysis. Drop-collapse test was performed in the polystyrene lid of a 96-micro-well plate. Cultures were tested in triplicate. The broth medium alone was a negative drop-collapse control. In oil displacement test, 15 µl of crude oil were added on the center of the oil film. The diameter and area of clear halo visualized under visible light were measured and calculated after 30 second. Also, the hemolytic activity of SL1 was screened on blood agar plates containing 5% (v/v) human blood.

**Effects of nickel (Ni$^+$) and zinc (Zn$^{2+}$) on SL1 growth.** Toxicity of nickel (NiSO$_4$) and zinc (ZnCl$_2$) determined by using methods of Hassen et al. (1998). Different concentrations of each metal prepared. The ranges of concentrations for heavy metals were 0, 1, 3, 5, 10, 25, 50, 100, 125 and 150 mM. Ion solutions were prepared in tubes with a final volume of 10 ml of nutrient broth (Merck, Germany). A metal-deficient Medium inoculated with the 200 µl of micro-organism. A metal-supplemented media without the bacteria (abiotic control) were used as negative controls. After 24 h incubation, bacterial growth was measured (OD 600 nm) (CE2021, U.K). Each assay was performed triplicate.

**Nucleotide sequence accession numbers.** The 16S rDNA sequences have been submitted to GenBank with accession numbers: strain SL1 (JQ996502)

## Results

**Physicochemical analysis of the water samples.** Na$^+$, K$^+$, Ca$^{2+}$ and Mg$^{2+}$ content of samples were 37500 ppm, 4500 ppm, 2400 ppm and 23741 ppm, respectively. The results showed that the water samples from the studied lake were saturated with Na$^+$ and contained high levels of Mg$^{2+}$. The pH of samples was between 7.13–7.49 and average of temperature at the sampling sites was 42°C.

**Phenotypic characterizations of the isolates.** The analysis of the isolates could be further identified them as part of a major *Firmicutes* group. The *Firmicutes* group related to the *Bacillaceae* family was represented by five strains. The results of phenotypic characterizations of isolates are shown in Table I. Among the isolates, SL1 grew well in wide range (up to 10%) of NaCl concentrations and 20–55°C of temperature. Optimum growth in wide range of salt indicated that the NaCl is not required for growth of SL1; therefore the isolate was regarded as a halotolerant *Bacillus*. Also this isolate produced three important hydrolytic enzymes in presence of 10% and 20% sea salt.

**Phylogenetic analysis.** Phylogenetic analysis based on 16S rDNA gene sequence comparisons revealed that the isolates SL2 and SL4 resembled to *Bacillus safensis* by 98% and 99% respectively. There were a 99% similarity between the isolate SL3 and *Bacillus pumilus* and 99.5% between the SL5 and *Bacillus sonorensis*. Also, the isolate SL1 fell within the branch encompassing members of the genus *Bacillus* and was related to *Bacillus licheniformis* with 97.0% 16S rDNA gene sequence similarity (Fig. 1). But SL1 isolate can be distinguished from *B. licheniformis* by physicochemical properties, 16S rDNA sequence and phylogenetic tree. Thus, suggested that the strain was not the known *Bacillus* and was tentatively named as *Bacillus* sp. SL1. This *Bacillus* considered as a unique microorganism for further study.

**Biofilm formation and biosurfactant production of SL1 isolate.** In Biofilm formation assay, optical density (OD) of eluted stain in 590 nm was 1.25 ± 0.31. This result indicated that the SL1 isolate have a strong (OD$_{590}$ ≥ 0.5) biofilm formation in present of 10% and 20% sea salt concentration. In semi-quantitative test for biosurfactant production the SL1 exhibited the high activity for oil displacement test toward Crude
Oil (3.14 ± 0.02 cm²) and clear haloes on blood agar plate. Also, this strain evaluated positive for drop-collapse test and emulsified crude oil in broth medium within 48 h of cultivation.

**Zinc and nickel resistance of SL1 isolate.** The ability of the SL1 to tolerate zinc and nickel was tested by tube method. The high concentrations of zinc in broth medium were effective on bacterial growth (Fig. 2). This strain exhibited average degree of tolerance to 1–25 mM concentrations of zinc (OD₆₀₀nm 0.480–0.141) and could live in medium with more than 25 mM zinc. On the other hand, nickel inhibited the growth of strain at very low concentrations. The lower optical density values revealed that the bacterial growth was affected due to the presence of metal in the growth medium (Fig. 2).

**Discussion**

Recent decades have seen a flow in studies on extreme environments including hypersaline ecosystems (Demergasso et al., 2004; Oren, 2002b). The hypersaline Lake Aran-Bidgol is located at an altitude of 800 m in an area with an arid to semiarid continental climate. It was formed by the deposition of halite sediments from an ancient sea in different geological periods (Makhoudoumi-Kakhki et al., 2011). According to the results of physicochemical analysis, sodium and magnesium concentrations of water samples were a high level, similarly to thalassohaline systems content. In addition, several interfering factors such as season, temperature, moisture and depth of sampling site could affect the ion concentrations of lake.

New halophilic Bacillus species from Aran-Bidgol saline Lake have been previously described. Recently Bacillus iranensis isolated from saline mud of this lake by Bagheri et al. (2012). In the present study, among the isolated strains, SL1 isolate was selected for further identification for its high halo-thermo tolerance and ability to produce most important industrial enzymes in present of 10% and 20% sea salt. Since most industrial procedure are performed under specific physicochemical conditions which may not be definitively adjusted to the optimal points needed for the activity of the existing enzymes; therefore, it would be of great importance to have enzymes that exhibit best possible activities at various ranges of salt concentration, pH and temperature. It is interesting to note that combined hydrolytic activity was detected in many halophilic strains (Rohban et al., 2009). Thus, these organisms are an excellent source of such enzymes that may be active at extreme conditions (Gomes and Steiner, 2004).

As determined by phylogenetic analysis, the SL1 isolate was closely related to the Bacillus licheniformis. But, according to the 16S rDNA sequencing and some physicochemical properties, SL1 was different from B. licheniformis that previously described. B. licheniformis is a Gram-positive, endospores forming and industrial organism that can be isolated from soils.
Halo-thermotolerant, biofilm-forming *Bacillus* sp.

and plant material all over the world (Veith *et al*., 2004). Moreover, there are reports that this organism is isolated from marine environments frequently (Ettoumi *et al*., 2009). But, to our knowledge, there is no report of *B. licheniformis* isolation from hypersaline Lakes, so far. Thus we tentatively named the SL1 isolate as *Bacillus* sp. SL1.

According to the results, SL1 isolate produced a strong biofilm in present of 10% and 20% sea salt. Also, this isolate exhibited tolerance to 1 mM to 25 mM concentration of zinc metal. A possible explanation for this ability is that SL1 isolate is protected in the environment conditions by using the biofilm formation. Based on previous investigations, the biofilm matrix has the potential to prevent diffusion of certain antimicrobial agents and heavy metal adsorption, thus restricting dispersion of compounds from the surrounding environment into the biofilm (Davey and Otoole, 2000; Maugeri *et al*., 2002; Tourney *et al*., 2009). Microbial biofilms contain bioorganic metal-complexing functional groups; thereby play an important function in metal cycling in contaminated environments. The results of Toner *et al.* (2005) study confirmed the importance of phosphoryl functional groups in zinc absorption by a bacterial biofilm. Also, there is a report of halophilic, thermotolerant *B. licheniformis* (B3-15), isolated from marine hot spring at Vulcano Island that was highly resistant to zinc (Maugeri *et al*., 2002).
On the other hand, the growth of SL1 isolate was inhibited at very low concentration of nickel. In contrast of our study, Kamika and Momba (2011) found a B. licheniformis-ATTC12759 could tolerate nickel at concentrations ranging between 1 and 2 mM. These observations of Bacillus sp. SL1 are in disagreement with previous reports of B. licheniformis so far and mostly confirmed that these different probably could be to special properties of various strains of Bacillus isolated from unusual environment.

In this study, the Bacillus sp. SL1 evaluated as a good biosurfactant producer similar to B. licheniformis strains that isolated from diverse locations (Maugeri et al., 2002; Yakimov et al., 1995). These microorganisms and their compounds can be used to enhance oil recovery, clean oil storage tanks, increase flow though pipelines reduce the heavy oil viscosity and stabilize fuel water-oil emulsions (Safary et al., 2010). Thus probably this microorganism can play a significant role in the bioremediation and treatment of industrial wastewater.

In conclusion on the basis of results, the present study could represent a new halo-thermotolerant Bacillus for remarkable amylase, protease, DNase, biosurfactant and exopolysaccharides in order to usage in bioremediation, biocatalysts production, medical and pharmaceutical industrial.

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**Literature**


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SHORT COMMUNICATION


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Abstract

The aim of the study was a retrospective analysis of the frequency of group B streptococci (Streptococcus agalactiae; GBS) carriage in pregnant women from the region of Krakow, together with an analysis of their drug resistance, carried out between 2008–2012. The study included 3363 pregnant women between 35 and 37 weeks of gestation, studied in accordance with the guidelines of the Polish Gynecological Society (2008). A high percentage of pregnant women who are carriers of group B streptococci was demonstrated. Each year covered by the study, it was in the range of 25–30%, with an average value equal to 28%. The results confirm the need for taking swabs from both the vagina and anus, since 15% of GBS-positive patients showed only rectal carriage. High percentage of isolates resistant to erythromycin was detected, which ranged from 22% to 29%, with an average value equal to 25%, as well as a high proportion of isolates resistant to clindamycin being 17–25%, with an average of 20%. The results indicate the need to standardize the methodology of collecting samples for GBS testing and introduce microbiological diagnostic standards in all gynecological and obstetric centers in Poland, in order to carry out a detailed epidemiological analysis in our country.

Keywords: Streptococcus agalactiae, antibiotic resistance, carriage of group B streptococci, pregnant women

Beta-hemolytic Group B Streptococci (GBS), represented by Streptococcus agalactiae, were the main etiological factor for neonatal infections in the USA in the 1970s. These infections usually appear in the form of Early Onset Disease (EOD), which develops in the first seven days of life with clinical manifestations of sepsis and mortality reaching 50%, or in the form of Late Onset Disease (LOD), which develops between the 7th and 90th day of life and usually takes the form of meningitis (Schrag et al., 2002; Verani et al., 2010).

The recorded rapid growth of the number of GBS infections in newborns was the reason behind devising guidelines aimed at newborn infections prevention by the American College of Obstetrics and Gynecology (ACOG) and Centers for Disease Control and Prevention (CDC) in 1996, and a year later by the American Academy of Pediatrics (AAP). CDC recommendations were updated for the first time in 2001 and published in 2002 (Schrag et al., 2002), and also subsequently subjected to evaluation and published once again in 2010 (Verani et al., 2010). Following the example of American scientists, many member countries of the European Union have introduced their own guidelines with the goal of preventing GBS infections in newborns. These include: Italy in 1996, Spain in 1998 and 2003, France in 2001, Germany in 1996 and 2008, Great Britain in 2003, Belgium in 2003, Switzerland in 2007 and the Czech Republic in 2008 (Rodriguez-Granger et al., 2012). In 2008, under the patronage of the Polish Gynecological Society (PTG), a Polish version of the recommendations was devised concerning the detailed instructions in the prophylaxis of GBS infections in newborns (Kotarski et al., 2008).

The acquired experiences indicate that the most effective method of limiting the number of infections in newborns is the introduction of screening of all pregnant women for GBS carriage and, should a positive result be obtained, implementing targeted perinatal antibiotic prophylaxis. It is a well-known fact that the most significant factor predisposing newborns to the development of infection is the presence of S. agalactiae in their mother’s genital or gastrointestinal tract, from where the bacteria are transmitted to the baby. Therefore, it is advised to carry out microbiological examination of

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the vagina and anus in women between the 35th and 37th week of pregnancy. As GBS colonization is often transitory, predicting on the grounds of a culture performed earlier than 5 weeks before delivery is fallible and recommended only in special cases, i.e. in women with a threat of premature labor or with premature rupture of membranes (PROM). If there is no test result for GBS carriage, the doctor should administer intrapartum chemoprophylaxis to a pregnant woman who has been diagnosed with at least one of the following risk factors of early onset disease: premature labor or PROM (before the 37th week of gestation); delivery at term, in which over 18 hours have passed since the rupture of membranes; developing fever of over 38°C for unknown reasons during delivery; developing urinary tract infection or bacteriuria caused by GBS during the course of the pregnancy; history of delivering a child, who was diagnosed with an infection of S. agalactiae etiology (Kotarski et al., 2008; Schrag et al., 2002; Verani et al., 2010).

In Europe, GBS colonization of pregnant women amounts to, according to literature data, from 6.6% in Greece (Tsolia et al., 2003), 7% in Spain (Bayó et al., 2002), 14% in Great Britain (Colbur et al., 2007), 16% in Germany (Brimi et al., 2006), 30% in the Czech Republic (Motlova et al., 2004) to 36% in Denmark (Hansen et al., 2004). In Poland, the percentage of GBS carriage in pregnant women, which is usually presented, differs significantly depending on the examined patient population and the employed method and comes to 4.3% in Lublin (Stupak et al., 2010), 5.13% in Łódź (Serafin et al., 2010), 11.4% in Warsaw (Kociszewska-Najman et al., 2010), 19% in Rzeszów (Krasniannin et al., 2008), 27.8% in Katowice (Romanik et al., 2011) and 30% in Krakow (Brzychczy-Włoch et al., 2012).

Getting to know the frequency of GBS carriage in pregnant women in various populations in Poland and the analysis of their drug resistance, observed over time, is of significant importance in epidemiological research and also in estimating the group of patients requiring the implementation of suitable antibiotic prophylaxis. Unfortunately, Poland is still lacking in sufficient data obtained with the use of uniform standards of microbiological examination assessing the frequency of GBS occurrence among pregnant women and their drug resistance. Furthermore, there is a lack of analyses depicting the dynamics of changes in carriage and drug resistance of S. agalactiae over the period of several years of research.

The objective of the work concerned a retrospective analysis of the frequency of GBS (Streptococcus agalactiae) carriage in pregnant women from the region of Krakow, as well as the analysis of their drug resistance, carried out in the years 2008–2012.

The testing of GBS carriage in pregnant women was conducted in the period of time from 1st January 2008 to 31st December 2012 on 3363 patients of the Rafal Czerwiakowski Na Siemiradzkiego Hospital in Krakow. The testing was carried out according to the PTG recommendations (Kotarski et al., 2008), upon prior acquisition of a positive opinion from the Bioethics Committee of the Jagiellonian University No. KBET/143/B/2007.

The research material was constituted by two samples taken from each pregnant woman with the use of separate cotton swabs, including a swab from the vagina taken without the use of a speculum and a swab from the anus taken after overcoming the sphincter resistance. The materials were taken between the 35th and 37th week of pregnancy, during a control visit to the gynecologist, and then, within 4 hours, delivered to the Microbiological Diagnostics Lab of the Jagiellonian University Medical College in Amies (bioMérieux) transport medium. Swabs were carried separately to Todd Hewitt Broth with the addition of gentamicin (8 μg/ml) and nalidixic acid (15 μg/ml) (Oxoid) and incubated for 18–24 h at 37°C in aerobic conditions. After preliminary pre-incubation, the materials were cultivated on Columbia Blood Agar (Difco) solid medium with the addition of 5% sheep blood and cultured for 24 h at 37°C in aerobic conditions. S. agalactiae species identification was carried out with the use of a SLIDEX STREPTO B (bioMérieux) latex agglutination test, CAMP test and API STREP (bioMérieux) test.

Drug resistance testing was performed for 1623 S. agalactiae isolates obtained from all of the positive materials, from both vaginal and anal swabs. The study was performed with the Kirby-Bauer antibiotic testing method using antibiotic discs: penicillin (10 IU), ampicillin (10 μg), clindamycin (2 μg), erythromycin (15 μg), nitrofurantoin (100 μg) and ofloxacin (5 μg) (Oxoid). The results were interpreted according to EUCAST guidelines (EUCAST 2012). There were three macrolide-lincosamide-streptogramin B resistance phenotypes determined for the S. agalactiae isolates, i.e. constitutive MLS resistance phenotype – cMLS, inducible MLS resistance phenotype – iMLS, and M phenotype.

The analysis of GBS carriage by year, with particular attention to the materials, i.e. anal swabs, was conducted by a χ² (chi-square) test. Meanwhile, the carriage in individual months was analyzed in two ways. The first one involved the employment of χ² test analysis; the second one, enabling more accurate investigations, consisted in conducting standardization of the fraction of GBS-positive patients for every month, in which the basis for standardization was determined by the average value and the standard deviation from the whole studied period. The dependence of standardized data on the months or seasons was tested using the analysis of variance and Student’s t-distribution test. Resistance trends in time were examined with the use of Pearson’s regres-
tion analysis. *P* values of < 0.05 were considered significant. Analyses were provided with package R 7.02.

In the analyzed period of five years (2008–2012), 3363 pregnant women were included into the study of GBS carriage. They were between their 35th and 37th week of gestation. The number of patients tested in 2008 was 620 (18%), in 2009 – 771 (23%), in 2010 – 802 (24%), in 2011 – 651 (19%), and in 2012 – 519 (16%). The studied group of patients was constituted by women between 18 and 44 years old with a average value age of 30.5. Altogether, 6726 materials, in the form of vaginal and anal swabs collected in pairs from each patient, were subjected to microbiological diagnostics.

The percentage of *S. agalactiae* carriers, determined in the consecutive years of the study, is presented in Figure 1. The place of isolation of streptococci, being either vagina, anus, or both simultaneously, is included. The obtained results point to a high percentage of GBS-colonized pregnant women and remained at a comparable level throughout the subsequent years. GBS carriage in the subsequent years was: in 2008 – 30% (184/620), in 2009 – 30% (233/771), in 2010 – 25% (202/802), in 2011 – 25% (189/651), and in 2012 – 28% (145/519), with the average value for the five-year period of the study reaching 28% (953/3363). It was demonstrated that the year of the study had no significant impact on the frequency of GBS carriage in pregnant women (*χ²* = 6.010; *p* = 0.1984).

The presence of *S. agalactiae* simultaneously in the vagina and anus was determined in 20% (670/3363) of women included into the study, which constituted 70% (670/953) of patients in comparison with the number of GBS-positive patients. The obtained results confirm the necessity to take anal swabs, as in 4% (147/3363) of the patients out of all of the pregnant women included into the research, GBS carriage was only present in anus, which constituted 15% among all the patients who were GBS carriers (147/953). On the basis of statistical analysis, a significantly higher detection of GBS carriage was demonstrated by taking into consideration the material, i.e. anal swabs (*χ²* = 16.365; *p* < 0.0001). Additionally, GBS carriage only in the vagina has been confirmed in 4% (136/3363) of patients, which constitutes 14% (136/953) in the group of GBS-positive patients.

The frequency of *S. agalactiae* carriage in individual months of the year that was analyzed over the period of 5 years encompassed by the research was presented in Figure 2. In selected cases, high divergence of results was obtained for individual months. It can be exemplified by two extreme values; the former being 15% (9/58) of patients colonized with GBS in May 2010 and over a threefold increase in the percentage of GBS-positive patients of 47% (35/47) in August 2009 (data not presented). The average percentage of carriage determined for individual months of the year amounted to: in January – 31%, February – 30%, March – 31%, April – 27%, May – 27%, June – 27%, July – 25%, August – 31%, September – 29%, October – 23%, November – 27%, December – 31%. In order to demonstrate whether there is a relationship between GBS carriage and the individual months, patient attendance was compared in the months encompassed by the research. No significant differences in GBS carriage were found while comparing individual months (*χ²* = 7.721; *p* = 0.7380). Similarly, the analysis of a relationship between the months and the standardized frequency of GBS carriage did not show statistical significance (*f* = 0.7141; *p* = 0.7190). However, since there were very many categories of

![Fig. 1. Frequency of *S. agalactiae* carriage in the vagina, anus and both of them simultaneously in the years 2008–2012.](image-url)
explanatory variables (months) in the analyzed sample with a relatively small number of measurements (years), showing such a significance is extremely difficult. For this reason, a generalization of the quantitative variable was performed changing it to seasons, what made it possible to demonstrate a difference on the border of statistical significance \((t = 1.940835; p = 0.0572)\). After removing the outlier, being August 2009 (data not shown), the observed difference was statistically significant for winter (the period from December to March), where the frequency of carriage was higher than with the other months of the year \((t = 2.163241; p = 0.0348)\).

While analyzing drug resistance, it was determined that all the \(S. agalactiae\) strains isolated in the period of five years encompassed by the study were sensitive to penicillin, ampicillin, nitrofurantoin, and ofloxacin. In the years 2008–2012, in the studied group of patients, the percentage of strains resistant to erythromycin was 7% (234/3363), while to clindamycin it was 6% (191/3363). Taking into the account the group of GBS-positive patients, the percentage of strains resistant to erythromycin in the years 2008–2012 reached 25% (234/953), in 2008 – 24% (45/184), in 2009 – 22% (51/233), in 2010 – 24% (49/202), in 2011 – 25% (46/189), and in 2012 – 29% (42/145). Similarly, in the case of clindamycin, the percentage of resistant strains in the years 2008–2012 was 20% (191/953), in 2008 – 21% (39/184), 2009 – 17% (39/233), 2010 – 19% (39/202), 2011 – 20% (38/189) and in 2012 – 25% (36/145). In the period of five years encompassed by the study, a resistance trend was determined showing statistically insignificant trend increase \((R^2 = 0.4653; f = 2.6115; p = 0.2045)\). It should be emphasized, though, that the time series involving only five years is inevitably susceptible to artifacts, so obtaining a fully reliable trend assessment would require a long time for analysis.

The percentage of isolates resistant to macrolides and the share of particular resistance phenotypes in the years encompassed by the research were presented in Figure 3. Among resistant isolates, in the years 2008–2012, the domineering one was the constitutive resistance phenotype for macrolides, lincosamides and streptogramins B \((cMLS_B)\), constituting 82% (191/234) among all of the resistant GBS isolates, followed by M phenotype 11% (26/234) and inducible phenotype \((iMLS_B)\) 7% (17/234).

In 2008, under the patronage of the Polish Gynecological Society (PTG) and on the basis of the CDC recommendations from 2002 (Schrag et al., 2002), a Polish version of the recommendations was devised concerning the detailed recommendations in the prophylaxis of GBS infections in newborns. In accordance with those, a test for GBS carriage in vagina and anus should be performed in pregnant women between the 35th and 37th week of pregnancy (Kotarski et al., 2008). According to the regulation of the Polish Minister of Health from 23rd September 2010 specifying the standards of conduct and medical procedures which accompany providing health services in perinatal care over a woman in the period of her philological pregnancy, childbirth, puerperium, and care over the newborn, it is recommended to perform vaginal vestibule and anal cultures for beta-hemolytic streptococci in all pregnant women between their 33rd and 37th week of pregnancy within the framework of the recommended range of prophylactic services and operations being part of health promoting services, diagnostic tests, and medical consultations (The regulation of the Polish Minister of Health from 23rd September 2010).
In November 2010, on the basis of the latest epidemiological data and the opinions of experts, CDC together with ACOG and AAP, and also with the American College of Nurse-Midwives, American Academy of Family Physicians as well as American Society for Microbiology, updated the guidelines from 2002 concerning preventing GBS infections in newborns. The most significant changes pertain to: (1) Expanding laboratory methods serving increasing the sensitivity of determining GBS in the studied materials by the employment of chromogenic culture media and the use of commercially-available tests based on nucleic acids amplification. (2) The introduction of screening of pregnant women for determining bacteriuria and establishing the threshold number of GBS amounting to ≥ 10^4 cfu (colony forming units) for 1 ml urine sample, for which the culture result is deemed positive. (3) Verification of conduct algorithm in the case of women with a threat of premature delivery or premature rupture of fetal membranes. (4) Defining the notion of suitable perinatal antibiotic prophylaxis, which consists in the employment of penicillin (changes in dosage), ampicillin or, additionally, cefazolin at least four hours before labor. (5) The notion of high risk of anaphylaxis was defined and, because of the high percentage of strains resistant to macrolides, erythromycin was withdrawn from perinatal prophylaxis. (6) In addition, the algorithm of conduct with a small newborn threatened with early onset disease (EOD) underwent modification (Verani et al., 2010).

By the employment of PTG recommendations from 2008 concerning the diagnostics of pregnant women for group B streptococci, a high percentage of *S. agalactiae* colonization of pregnant women was confirmed on the basis of the obtained results. The number, in the individual years encompassed by the study (2008–2012), ranged from 25% to 30%, with the average value of 28%. This result brings Poland closer to the countries with the highest percentage of GBS colonization among pregnant women that is described in literature, i.e. Slovakia with 30% (Motlova et al., 2004) and Denmark with 36% (Hansen et al., 2004). In comparison with our previous research, from the years 2004–2006, in which carriage, depending on the applied method, was estimated to range between 13% and 17% (Strus et al., 2009) and from the years 2007–2009, in which the participation of GBS-positive patients reached 30% (Brzychczy-Włoch et al., 2012). The current result points to a lingering high percentage of colonized pregnant women residing in the region of Krakow. The observed higher incidence of carriage was most probably connected with the increase in sensitivity of the diagnostic methods and the introduction of standardized microbiological procedures. Hansen et al., 2004, reached similar results upon the introduction of a new differential medium, by which the sensitivity of GBS detection was increased from 15% to 36% (Hansen et al., 2004). The detected high percentage of GBS colonization in the studied group of patients differs significantly from the results obtained by researchers from other centers in our country. For instance, the research of the Warsaw center carried out in the years 2007–2008 recorded GBS colonization in 11.4% of pregnant women (Kociszewska-Najman et al., 2010); similarly, the results of the Łódz center from 2008 indicate only 5.13% carriage in that patient population (Serafin et al., 2010) and the research

![Fig. 3. Percentage of *S. agalactiae* strains with cMLS, iMLS and M resistance phenotypes in the years 2008–2012.](image-url)
performed in 2008 by the Lublin center, in which GBS carriage was demonstrated only in 4.3% of women (Stupak et al., 2010). The low percentage of colonization described by the authors of the quoted studies might have been related to different geographical areas and methodology of the conducted research. For instance, in the research carried out in Lublin, the studied material was constituted only by vaginal swabs and there is a lack of a description of culture methodology and diagnostics of group B streptococci (Stupak et al., 2010). Worth noting to the special resemblance of our results to the data coming from Katowice, where percentage of GBS colonization in pregnant women was 27.8% (Romanik et al., 2011).

It is worthwhile mentioning that the standardization of microbiological diagnostics with the aim of determining GBS carriage in women is of fundamental importance in correct diagnostics of pregnant women. In accordance with CDC, the lack of preliminary material preincubation in a selective medium decreases method sensitivity even by 50% (Schrag et al., 2002). Likewise, collecting swabs only from the vagina or only from the anus influences obtaining false-negative results in as many as 30% of patients (Brimil et al., 2006). The statistically significant results confirm the necessity to take anal swabs in parallel with vaginal swabs, as 15% of patients displayed carriage only in anus. Hence, the omission of the diagnostic material constituted by anal swabs has an impact on obtaining false-negative results in the amount of as much as 15%. Brimil et al., 2006, obtained similar results, which prove the fact that taking only vaginal swabs results in getting false-negative results in 24% of cases (Brimil et al., 2006). Summing up, according to the current PTG recommendations from 2008, there are several factors significantly influencing a correct test result for GBS carriage, i.e. the type of material collected, it is required to be a vaginal and an anal swab, which, after their collection, can be put together into one test tube; employing appropriate transport media, e.g. Amies medium; preliminary 18–24 h material preincubation, often skipped in many labs, which can be carried out, for example, on Todd Hewitt Broth with an addition of suitable antibiotics hampering the proliferation of other bacteria; isolation of all colonies characteristic of streptococci from the medium with blood, even the ones which do not possess beta hemolysis, since there is a small proportion of S. agalactiae strains that do not display this feature (Kotarski et al., 2008; Schrag et al., 2002; Verani et al., 2010).

A detailed analysis of the dynamics of change for individual months of the calendar year pointed to a statistically significant increase in carriage in winter months, in comparison to the remaining parts of the year. Ma et al., 2012, in the research concerning pregnant women from Taiwan, demonstrated an increase in frequency of isolation of GBS strains representing hypervirulent ST-17 clone in winter months (Ma et al., 2012). On the other hand, the study carried out by Dadvand et al., 2011, indicates a statistically significant relation between the frequency of GBS carriage and August, the warmest and most humid month in Spain (Dadvand et al., 2011). The obtained result is difficult to discuss as there are no publications describing analyses of this kind carried out on a similar sample group residing in a geographical area comparable as regards the climate.

Even though penicillins have been used for many years now, most of the S. agalactiae strains are still sensitive to this antibiotic (Schrag et al., 2002; Verani et al., 2010). However, the first strains of GBS with reduced penicillin susceptibility (PRGBS) were characterized in Japan (Kimura et al., 2008). Our research results confirm the sensitivity to penicillin and ampicillin for all of the GBS strains isolated in the period of five years encompassed by the study. However, in recent years, GBS strains’ resistance to macrolides, lincosamides and streptogramins B is more and more often described. Uh et al., 2005, demonstrated significant increase in the frequency of isolation of GBS strains resistant to erythromycin, from 0% in the 1990s to 41% in 2002, and to clindamycin, from 0% to 48% in the same periods (Uh et al., 2005). In Europe, the percentage of GBS strains resistant to erythromycin is from 11% in Germany (Schoening et al., 2005), 14% in Spain (Gherardi et al., 2007), 16% in Italy (Gherardi et al., 2007), 21% in France (De Misy et al., 2001), to 22% in Turkey (Acikgoz et al., 2004). The high percentage of S. agalactiae strains resistant to erythromycin reaching 25% and the high proportion of isolates resistant to clindamycin reaching 20%, were recorded by us, confirm the worldwide tendencies, indicating increase in GBS drug resistance. Two mechanisms are responsible for the resistance of streptococci to macrolides. The first one consists in active removal (pumping out) of the antibiotic from a bacterial cell. This mechanism is marked with phenotype M, coded by mef gene and determines resistance to 14- and 15-membered macrolides, i.e. erythromycin, clarithromycin, roxithromycin and azithromycin, but resistance to 16-membered macrolides, e.g. spiramycin and clindamycin. The second type of resistance stems from methylation in place of the target activity in the ribosome and conditions the resistance to all the macrolides, lincosamides, and streptogramins B, which is described with MLS\_b phenotype of constitutive or of inducible character; however, clinically, it indicates a lack of effectiveness of all of those drugs (cross-resistance) (Acikgoz et al., 2004; Uh et al., 2005). In the isolate pool studied by our team, the constitutive phenotype was the dominant one (82%), followed by phenotype M (11%) and the inducible phenotype (7%),...
at the same time, the share of particular phenotypes was different in the individual years of the research.

To sum up, in the light of literature data from our country, often making use of different methods for diagnosing GBS from the recommended ones and also using the standards modified in 2010 by CDC, it is necessary to consider an update of the 2008 PTG recommendations and publishing them again, following the example of other countries, under the patronage of all of the Polish societies, directly related to the problem of newborn infection prophylaxis.

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Literature


The regulation of the Polish Minister of Health from 23rd September 2010. Annex to the Regulation – Standards and procedures for the award of medical health services in the field of perinatal care exercised over women during physiological pregnancy, physiological childbirth, puerperium and infant care (in Polish).


A Case of a Late and Atypical Knee Prosthetic Infection by No-Biofilm Producer Pasteurella multocida Strain Identified by Pyrosequencing

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Abstract

Prosthetic joint infections due to Pasteurella multocida are rarely but increasingly reported but no data on production of biofilm are available. We report the case of a woman with a late, haematogenous peri-prosthetic infection of cemented total knee arthroplasty caused by a strain of P. multocida identified by pyrosequencing and unable to produce biofilm. Comparison of clinical and laboratory findings with those reported in other patients evidenced differences mainly in the period of symptoms’ onset and in the behaviour of some inflammatory markers.

Keywords: Pasteurella multocida, bacterial biofilm, prosthetic joint infections

A 82-year-old woman was referred to our department in May 2010 because of an acute onset of a PJI of her left total knee cemented prosthesis, implanted four years before (Fig. 1A). In July 2000 a total knee replacement had been implanted to the patient for severe knee rheumatoid arthritis; early revision of the wound was required, due to post-surgical bleeding. Asymptomatic for the following years, she underwent femoral component revision for “aseptic loosening”. In May 2010 she presented to our observation with a swelling, painful and warm knee with functional limitation and a draining fistula in the anterior aspect of the mid-leg. Clinical history revealed cat scratches over the homolateral foot and ankle five months before, followed by local erythema, that resolved after a short antibiotic course administered by the general practitioner (amoxicillin-clavulanic acid 1 g twice a day for ten days). At admission to our hospital, C-reactive protein (CRP) was 7.67 mg/dL, Erythrocyte Sedimentation Rate (ESR) 120 mm/hr, white blood cells count 8.3 × 10³/uL and haemoglobin level 9.7 g/dL. Plain x-ray showed no sign of prosthetic loosening (Fig. 1B). Cultures from the fistula were negative. In June 2010 surgical debridement with change of the mobile parts of the prosthesis (polyethylene tibial tray) and intra-operative cultural examination was performed both at the joint and at
the tip of the tibial prosthetic stem, where there was the draining fistula (Fig. 1C and 1D). All six intra-operative samples yielded *P. multocida*, identified by biochemical methods (API NH, Biomerieux Nany l’Etoile, France) and subsequently confirmed by Pyrosequencing (PSQ96RA, Diatech, Jesi, Italy) of three regions of the 16S ribosomal gene, as previously reported by Jonasson et al. (2002). The obtained sequences were compared with bacterial 16S rDNA sequences available at NCBI using the BLAST advanced options tools including taxonomy and lineage reports (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi). Isolated *P. multocida* proved susceptible to β-lactams, fluoroquinolones and tetracycline. Histological examination of the joint capsule and peri-prosthetic tissues revealed a chronic synovitis with more than 10 leukocytes per field, compatible with infection. Pre- and post-operative IL-6 concentrations were 12.37 and 14.42 pg/ml respectively and 6.89 pg/ml at 5 days after surgery. Screening of biofilm production was investigated by an *in vitro* spectrophotometric method as previously described by Christensen et al., 1985. The amount of biofilm was quantified by reading optical density (O.D.) at a wavelength of 595 nm. The assay was performed in duplicate and repeated for three times. Amount of biofilm produced by the isolate was compared with that produced by another strain of *P. multocida* from our collection and the ATCC strain 6529, which had been previously characterized for their production of biofilm and used as positive controls.

Spectrophotometric analysis evidenced no production of biofilm by the human isolate differently by the positive controls (O.D. 0.256 vs 1.610 and 1.242 respectively). In order to confirm the spectrophotometric assay, we have tested through confocal laser scanning microscopy (CLSM), the ability of our strain to produce biofilm on prosthetic material. Briefly, sandblasted titanium disks with 20 mm diameter and 6 mm thick were used as a substrate for the biofilm formation while, FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Invitrogen, Ltd. Paisley, UK) is the staining used for bifilm. We used a confocal laser scanning microscope Leica TCS SP5 CLSM, opting for a three-dimensional z-stack acquisition (in a solid field described by the axes xyz) of five different portions for each disk. Sections were acquired in succession along the z-axis for a total of 150 µm thickness. Acquired images during the experimental session were processed through a segmentation algorithm (Volocity; Perkin Elmer, Waltham, MA) capable of separating the signal from the background and the sample, in order to obtain a proportionality between the number of bacteria and the fluorescent signal. This analysis confirms the previous screening test because, even in this case, it demonstrates how the clinical strain isolated not produce biofilm (Fig. 2B) compared to positive controls (Fig. 2A). The patient was

![Fig. 1. Panel A: Clinical presentation in May 2010 of the acute, late, haematogenous infection of the left cemented knee joint prosthesis; Panel B: Antero-posterior and lateral X-ray views at the time of infection occurrence; Panel C: Intra-operative evidence of peri-prosthetic infection and osteolysis at the joint level; Panel D: Mid-shaft tibial bone fistula in communication with the skin.](image1)

![Fig. 2. 3D reconstruction of *P. multocida* biofilms by CLSM. Biofilms were stained with FilmTracer™ LIVE/DEAD® Biofilm viability kit: SYTO9 (green) represents the viable cells; Propidium iodide (red) represents the dead cells. Panel A: *P. multocida* ATCC strain 6529 (Positive control); Panel B: Clinical strain isolate from patient.](image2)
treated with a combination of amoxicillin-clavulanic acid 1 g three times a day for 34 days and ciprofloxacin 750 mg per day for 42 days, according to bacterial antibiotic susceptibility pattern. One week after surgery the patient was transferred to the rehabilitation unit of our institute. She remained there for 15 days. When she left the hospital, her leukocyte count was $4.5 \times 10^9/\mu L$, haemoglobin level was 8.9 g/dL, CRP 4.3 mg/dL, procalcitonin <0.5 ng/mL and IL-6 was 4.35 pg/mL. Eighteen months after surgery, there are no clinical signs of infection recurrence, CRP and ESR had returned to baseline levels.

Only few cases of PJIs, caused by *P. multocida*, have been described over the last two decades (Antuna et al., 1997). Known risk factors for *P. multocida* infections include age, diabetes, rheumatoid arthritis, obesity, immunosuppression, previous surgical interventions and renal insufficiency (Heym et al., 2006). A part from rheumatoid arthritis and age, no other risk factors known to be associated to *P. multocida* infection were recognized, and clinical history did not evidence other predisposing conditions.

As in our case, in all the previously reported ones, the infection occurred late after joint prosthesis implant and a distal source was identified. However, differently from what observed in other cases, symptoms arose rather far from the cat bite (5 months vs a maximum of 2 months) and did not include fever. The lack of fever as well as the moderate increase in CRP and leukocyte count in respect to the other reported cases (7.67 mg/dL vs 27.7 mg/dL and $8.3 \times 10^9/\mu L$ vs $14.6 \times 10^9/\mu L$) could suggest a clinical picture of masked effect of the immunocompromised state of the host as affirmed by Metha and Mackie (2004) but, in this case, we did not find parameters confirming an altered immune status of the patient. For this reason, we hypothesized that the moderate elevation of inflammatory parameters could be due to the longer time elapsed from the cat bite in respect to what has been reported by other authors (Heym et al., 2006; Metha and Mackie, 2004). Maradona et al. (1997) advise that antibiotic prophylaxis would be prudent in patients who have suffered a pet bite or scratch and have a prosthetic joint. One- or two-stage revision surgery is recommended in cases of septic implant loosening. In acute, late haematogenous infection, surgical debridement with prosthesis retention may be attempted as a salvage procedure, with a success rate < 80% (Heym et al., 2006). In particular, according to Heym, surgical debridement and antibiotic therapy, without removal of an infected TKA, are indicated when the infection is acute, the responsible microorganism has been isolated, the organism is susceptible to oral antibiotics, antibiotics could be tolerated without serious toxicity, and the prosthesis was not loose (Heym et al., 2006). Old age and the presence of a well fixed, long-stemmed, cemented revision joint prosthesis, whose removal is particularly challenging and associated with severe bone loss, were the other factors that prompted us to try a salvage procedure of the implant in this particular case.

The role of biofilm in PJIs is well known and its presence has been related to chronic infections and to difficulties in their eradication. Ability of *P. multocida* to produce biofilm has been rarely evaluated. Available studies indicate *in vitro* production of biofilm by strains of animal origin (Olson et al., 2002). Nonetheless, it may be hypothesized that the ability to produce biofilm might be different *in vitro* and *in vivo*, where host factors may favor its formation. However, comparison with the positive control strains clearly indicated the absence of biofilm production by the tested isolate.

The role of PCT in diagnosis of PJIs is matter of debate, since no clear evidences of its usefulness have been provided. In a recent study, PCT was found to be of no value in differentiating septic loosening (Drago et al., 2011) and this patient confirmed these results. In contrast, IL-6 levels remained higher than the normal range in the preoperative and postoperative phases, confirming its reliability as useful marker of prosthetic infection.

In the light of our result, the observed differences compared to other cases, highlight the importance of measuring alternative inflammatory parameters in diagnosing prosthetic infection.

Although obtained in an isolated case and at a relatively short follow-up, considering biofilm production as a potent virulence factor for bacteria that colonize implanted biomaterials, one may speculate if implant-related infections, caused by no-biofilm producing bacteria, may be treated with systemic antibiotic and prosthesis retention with a better prognosis than implant-related infection associated with biofilm producing bacteria.

**Literature**


Short Communication

**Application of a Real-Time PCR Method for Salmonella spp., Escherichia coli, Staphylococcus aureus and Clostridium perfringens Detection in Water Samples**

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**Abstract**

The diagnostic assessment of water sanitary state is based mainly on the cultivation of bacteria retained on membrane filters. However classical microbiology methods have a lot of disadvantages. More and more frequently, rapid detection and identification of pathogens present in water is based on molecular biology techniques. The aim of this study was to determine the effectiveness and usefulness of a real-time PCR method, when compared to the recommended bacteria culture method, in diagnostics of pathogens in water samples. The research concerned the detection and identification of main sanitary indicators of water such as: Salmonella spp., Escherichia coli, Staphylococcus aureus and Clostridium perfringens. The analyses were conducted in water samples contaminated with the reference material (the aforementioned bacteria) and real environmental samples, which were examined for the presence of nucleic acid of: Salmonella spp., E. coli, S. aureus and C. perfringens using a real-time PCR method.

**Keywords:** C. perfringens, E. coli, Salmonella spp., S. aureus, real-time PCR, water

Water is not considered to be the habitat of pathogenic bacteria, such as: Escherichia coli, Salmonella species, Clostridium perfringens. Usually, the microorganisms are transferred there directly from human or animal bodies. Sewage can also be a direct source of pollution. Contact with contaminated water may be hazardous for human health. These bacteria are basic indicators of sanitary contamination of surface water, as well as bathing and drinking water.

*E. coli* and *Salmonella* spp. are Gram-negative bacteria of the family Enterobacteriaceae. The first one, *E. coli*, partly forms physiological flora of human and animal colon, but in specific conditions it may become pathogenic (e.g. when bacteria migrate to systems other than the intestine or when they produce toxins). Infections in children and adults manifest themselves by watery diarrhea. Less common symptoms of diarrhea are fever, nausea, abdominal pain, stool with blood and mucus (Cennimo et al., 2007). The virulence of *E. coli* strains depends on the adhesion to the gastrointestinal epithelium, and the production of toxins (Chattaway et al., 2011, Kaur et al., 2010). *Salmonella* is the main cause of food poisoning, typhoid fever and paratyphoid fever. Mostly, it is transmitted to humans by contaminated food and water (Ohl and Miller, 2001).

*C. perfringens* is a Gram-positive, anaerobic, rod-shaped bacterium producing endospores. The gastrointestinal tract of animals and humans is its habitat. *C. perfringens* strains were classified into five types based on the ability to produce four toxins: α, β, ε, ι (Cavalcanti et al., 2004; Kądzielńska et al., 2012).

*S. aureus* is a Gram-positive bacterium of Staphylococcaceae family which is found in the upper respiratory tract, on the human skin and the urogenital system. In specific conditions, *S. aureus* may cause diseases such as: acute skin infections, subcutaneous tissues and soft tissues infections, systemic infection, and infection or poisoning associated with the production of toxins. The symptoms of such poisoning are diarrhea, nausea, low blood pressure, septic shock, and even death. The presence of this bacterium does not always entail infection. A large number of humans turn out to be asymptomatic carriers of the bacterium (Bien et al., 2011).

The diagnostic assessment of water sanitary state is based mainly on the cultivation of bacteria retained on membrane filters. The major disadvantage of this...
cultivation method is long waiting time for the results. Moreover, it is often limited by composition of a medium used to grow a particular culture. Some pathogens may grow faster on a particular medium, which inhibits the growth of others (Toze, 1999). Medium ingredients provide microorganisms with conditions approximately similar to natural ones (Lee et al., 2006). However, the methods of classical microbiology are inexpensive and uncomplicated (Rogers et al., 2011). Currently, molecular biology techniques based on polymerase chain reaction (PCR) are used in microbiological diagnosis. They enable rapid detection and identification of all pathogens present in water (Valasek and Repa, 2005; Wong and Medrano, 2005). The most widespread molecular method of diagnosis, real-time PCR (quantitative PCR, qPCR), is characterized by high sensitivity, specificity, efficiency and quickness (Wong and Medrano, 2005; Yang et al., 2002; Smith and Osborn, 2008). The application of a real-time PCR method enables to identify genetic material of microorganisms directly from environmental samples (Kacprzak et al., 2012; Toze, 1999). Unfortunately, the detected genetic material can be derived from both dead and living cells (Fey et al., 2004). The risk of contamination in a real-time PCR method is very small, whereas the capacity of this method is very high (Dixit and Shaker, 2009; Heid et al., 2012). The unquestionable advantage of a real-time PCR method is the ability to detect living bacteria that are dormant. These bacteria are not capable of growing on microbiological media (VBNC, viable but nonculturable) (Rogers et al., 2011; Toze, 1999; Dufour and Stelma, 2010).

The aim of this study was to determine the effectiveness and usefulness of a real-time PCR method for identification of pathogenic bacteria which are found in water samples: Salmonella spp., E. coli, C. perfringens and S. aureus.

In the first stage of the experiment, the detection limit of tested pathogens was determined. Reference materials EasiTab™ (BioSenate, Bury, UK) containing lyophilized microorganisms: E. coli – NCTC 9001, C. perfringens – NCTC 8797, S. aureus – NCTC 6571 and reference material MicroPellet (Meconti, Mondorf-les-Bains, Luxembourg) containing a lyophilized strain of S. enterica – ATCC 14028 were used to produce stock solutions of bacteria. Each solution had a volume of 100 ml and contained the following number of microorganisms: E. coli – 2.1 × 10^7 colony forming unit (CFU), C. perfringens – 6.9 × 10^4 CFU, S. aureus – 3.0 × 10^4 CFU, S. enterica – 6.2 × 10^4 CFU. The dilution series of contaminated samples at concentrations ranging from 10^6 to 10^-4 were prepared. Sterile water was used for dilution. Each dilution contained a known number of microorganisms. This helped to define the limits of detection in a real-time PCR method. Extraction of DNA and pathogen detections in individual dilutions were conducted using methods and reagents described below.

Then, contaminated samples were prepared using reference materials: EasiTab™ and MicroPellet. Lyophilized bacteria was dissolved in sterile water according to the manufacturer’s instructions. There were fifteen contaminated samples. Each solution had a volume of 1500 ml. In addition, twenty-five blank samples (sterile water) were prepared (Table 1). All samples were duplicated. One part was analyzed using a real-time PCR method, and the other using cultivation methods. Moreover, fifty environmental samples of water were obtained for analysis. Environmental samples were collected from surface sources of water (streams, rivers, lakes), swimming pools, deep water intakes, surface water intakes and mixed water intakes. Each sample had a volume of 1500 ml and was duplicated. One part was analyzed using a real-time PCR method and the other using cultivation methods.

At the beginning of a water sample analysis, DNA extraction from bacteria was performed with a commercially available kit Aqua Screen® FastExtract (Minerva Biolabs, Berlin, Germany). The whole volume of water samples was filtered through membrane with a pore size of 0.45 μm, which was attached to the kit. This step was identical to the steps of water samples preparation for cultivation methods. The next stages were prepared according to the manufacturer’s instructions, however cell lysis was modified. The whole volume of lysate was used for incubation at 56°C, which resulted in sensitivity increase and increased recovery of the genetic material from the filter.

The detection of pathogens in contaminated and environmental samples was performed using LightCycler 480 II (Roche, Basel, Switzerland). The amplification reactions were performed using commercially available kits: SureFood BAC Staphylococcus aureus PLUS LC (Congen Biotechnology, Berlin, Germany), SureFood BAC Clostridium perfringens PLUS LC (Congen Biotechnology, Berlin, Germany), Foodproof Salmonella Detection Kit (Biotecon Diagnostics, Potsdam, Germany), Foodproof E. coli and Shigella Detection Kit (Biotecon Diagnostics, Potsdam, Germany). The thermal profile of Salmonella spp. and E. coli analysis for a real-time PCR was as follows: pre-incubation at 37°C for 2 min, initial denaturation at 95°C for 10 min, and then 45 cycles consisting of the following temperatures and time intervals: 95°C for 5 s, 59°C for 35 s and 72°C for 15 s. The last step was cooling at 40°C for 30 s. Whereas, the thermal profile of S. aureus and C. perfringens analysis was as follows: denaturation at 95°C for 5 min, and then 45 cycles consisting of the following
temperatures and time intervals: 95°C for 15 s, 60°C for 15 s. All assays were conducted in duplicate.

The crossing point (Cp) values were determined automatically using the Second Derivative Maximum method. The results interpretation in Salmonella spp. and E. coli analyses was conducted in the Red 640 channel (excitation wavelength/emission wavelength: 498–640 nm) while internal control was analyzed using the Cy 5/Cy 5.5 channel (excitation wavelength/emission wavelength: 498–660 nm). The FAM channel (excitation wavelength/emission wavelength: 465–510 nm) was used for S. aureus and C. perfringens analyses while internal control was analyzed using ROX/Texas Red channel (excitation wavelength/emission wavelength: 533–610 nm).

The study of water using reference methods of classical microbiology was performed to demonstrate the growth on selective media. Microbiological studies were conducted according to the test procedures based on the standards and regulations of the National Institute of Public Health – National Institute of Hygiene in Warsaw.

All contaminated samples were analyzed by a real-time PCR method for the presence of Salmonella spp., E. coli, S. aureus and C. perfringens. The increase of fluorescence was observed in contaminated samples, whereas no increase of fluorescence was observed in blank samples (data not showed). The positive results of tested pathogens detection were characterized by a logarithmic increase of fluorescence. The Table II shows Cp values for particular analyses. The variation coefficient of Cp values was 1.52%. The assessment of reaction parameters shows that the amplification was conducted correctly.

The results obtained by a real-time PCR method were confirmed by the cultivation method. In this study, fifteen contaminated positive samples and twenty-five negative samples were obtained using a molecular method and a classical method of microbiology (Table III). The detection limits were determined for each pathogen using a dilution series of known concentrations. The detection limit was respectively: Salmonella spp. – 6.2 × 10² CFU, E. coli – 2.1 × 10⁴ CFU, S. aureus – 3.0 × 10⁵ CFU, C. perfringens – 6.9 × 10⁴ CFU. Apart from detection limit, other validation parameters of real-time PCR method were collected in the Table IV. The additional analysis of validation parameters confirmed studies and results reliability.
The examination of fifty environmental water samples by reference cultivation methods and an alternative molecular biology method revealed differences. In three samples which were negative in the cultivation method, the presence of \textit{S. aureus} genetic material was confirmed (Table III).

Urbanization and industrialization result in water pollution in the developed and developing countries. Water can be the source of pathogenic bacteria which are responsible for spreading infections in the whole world (Dixit and Shaker, 2009). To avoid this, you need to improve diagnosis of these organisms. Many published reports describe using real-time PCR techniques to facilitate the identification of pathogens detected in various environmental samples (Ibekwe and Grieve, 2003; Ahmed et al., 2008; Lee et al., 2006).

The results obtained in this study confirmed the importance of a real-time PCR method for a fast diagnosis of pathogens which are present in water. For all tested pathogens such as: \textit{Salmonella} spp., \textit{E. coli}, \textit{S. aureus} and \textit{C. perfringens} effectiveness of the method was confirmed. The results obtained by a real-time PCR assay were confirmed by a cultivation method. This is important because it provides a high quality of diagnosis and accuracy of analyses. The differences between the results of testing environmental samples resulted from restrictions of a cultivation method, which was due to a random choice of representative bacterial colonies.

Application of a real-time PCR assay allowed to reduce significantly the waiting time for results. When every minute counts, it is very important to confirm or rule out the possibility of bacterial infection. The isolation of genetic material without a pre-multiplication reduces the identification time of pathogens which are present in water. Aqudelo et al. (2010) draw similar conclusions on the cultivation method and a real-time PCR assay in \textit{E. coli} and fecal enterococci diagnosis from water samples. Rintilla et al. (2011) analyzed stool samples using the same method.

Molecular biology techniques have many opponents, and cultivation methods are the "golden mean" in the water diagnosis. In recent years, a lot of molecular techniques have been approved for the detection of pathogens, but unfortunately there are still difficulties such as: lack of standardization, reproducibility and stability of the isolated genetic material (Beneduce et al., 2007, Noble and Weisberg, 2005). However, it can be of great significance to quickly obtain the results of pathogens detection in water. This allows the sanitary services to respond immediately and suppress the public health problem almost at the beginning.

Summarizing, this paper confirmed the efficacy of a real-time PCR method in the diagnosis of the presence of pathogenic bacteria in water. Moreover, a real-time PCR can be an important tool for fast and specific microbiological diagnosis.

<table>
<thead>
<tr>
<th>Object of study</th>
<th>Positive samples number</th>
<th>Negative samples number</th>
<th>Percentage of certified results (%)</th>
</tr>
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<tbody>
<tr>
<td>Contaminated samples of water</td>
<td>15</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Environmental samples of water</td>
<td>3</td>
<td>47</td>
<td>94</td>
</tr>
</tbody>
</table>

Table III

<table>
<thead>
<tr>
<th>Results correctness [%]</th>
<th>\textit{Salmonella} spp.</th>
<th>\textit{E. coli}</th>
<th>\textit{S. aureus}</th>
<th>\textit{C. perfringens}</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC* = 100</td>
<td>AC* = 100</td>
<td>AC* = 100</td>
<td>AC* = 100</td>
<td>AC* = 100</td>
</tr>
<tr>
<td>SE* = 100</td>
<td>SE* = 100</td>
<td>SE* = 100</td>
<td>SE* = 100</td>
<td>SE* = 100</td>
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<tr>
<td>SP* = 100</td>
<td>SP* = 100</td>
<td>SP* = 100</td>
<td>SP* = 100</td>
<td>SP* = 100</td>
</tr>
<tr>
<td>Detection limit [CFU]</td>
<td>6.2 \times 10^3</td>
<td>2.1 \times 10^3</td>
<td>3.0 \times 10^3</td>
<td>6.9 \times 10^3</td>
</tr>
<tr>
<td>Repeatability [%]</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Reproducibility [%]</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Method uncertainty [%]</td>
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<td>1.8</td>
<td>1.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Relative accuracy [%]</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Relative sensitivity [%]</td>
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<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Relative specificity [%]</td>
<td>100</td>
<td>100</td>
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<td>100</td>
</tr>
</tbody>
</table>

Table IV
Basic parameters of the validation of real time PCR method.

* AC – accuracy, SE – sensitivity, SP – specificity
Literature


The Influence of the Organic Matter of Sewage Sediments on Biological Activity of Microorganisms which Carry out the Transformations of Carbon and Nitrogen Compounds

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Abstract

Soil microorganisms play an important role in the organic matter transformation process. The soil microorganisms also are in symbiotic relationship with plants. At the same time, soil microorganisms are sensitive to both anthropogenic and natural habitat changes. Particular characteristics of organic matter (the C:N relation, pH, the content the content of assimilated nutrients, the xenobiotics etc.) modify the biotic conditions of the soils. This particularly concerns the microorganisms which carry out the changes in the mineral and organic nitrogen compounds and the transformation of the external organic matter. The first aim of this work was to assess the influence of the sewage sediments and the manure on the phytosanitary potential of the soil environment. The second aim of this article was to estimate the number and activity of microorganisms which carry out the transformation of carbon and nitrogen compounds. This work showed the stimulating effect of the external organic matter both on the number and on the activity of most of the physiological groups. The manure mainly stimulated ammonificators, amylolitic microorganisms and Azotobacter sp. The sewage sediments mainly stimulated ammonificators, nitrifiers of I phase and cellulolytic microorganisms. The statistically significant impact of the physio-chemical soil habitat on the biological activity of the analyzed groups of microbes was also noted.

Key words: biological activity, microorganisms, nitrogen fixation, organic matter

The content of organic matter determines the significant fertility rate and the usefulness of the soil. In scientific literature (Fernandes et al., 2005; Pisarek, 2007; Pisarek et al., 2012, Wilkinson et al., 2003), a reduction in the amount of humic compounds was often observed. This reduction may indicate unfavorable changes in the balance of the soil carbon and nitrogen compounds. External organic matter in the form of sewage sediments added into the soil could bring many advantages to the soil environment. Those involved in the agricultural use of these materials, however, must also take into account the need to control and eventually reduce the pollution and toxic components which could be accidentally included in the trophic chain. Farm use of manure and sewage sediments is one of the most economical form of their utilization (Lee and Liu, 2002; Wang et al., 2004). There is a high content of assimilated forms of nitrogen and phosphorus compounds in sewage sediments. Thus, sediments are very helpful for agricultural use as valuable soil conditioners (Pisarek, 2007; Pisarek et al., 2012; Sohaili et al., 2012; Wilkinson et al., 2003). Injecting sewage sediments into the soil might also cause changes in the number of microorganisms or in the intensity and direction of the physiological processes carried out by the microorganisms (Akiyama et al., 2004; Jezierska-Tys et al., 2004; Joniec and Furczak 2012; Li et al., 2009; Piontek and Lonc, 2000). The biomass of the soil microorganisms is only a little bit more than 5% of the organic matter in the soil. Nonetheless, the microorganisms have a big impact on the quality of the soil and the productivity of the ecosystems. Microorganisms release hundreds of different kinds of enzymes into the soil environment. In the transformation of the external organic matter, the most important enzymes are the ones, which participate directly in the degradation of the lignin and cellulose degradation, and the enzymes which take part in the mineralization and the circulation of nitrogen, phosphorus and sulphur. The degradation of polymers requires multi-component enzymatic systems which are produced by various groups of organisms. Nitrogen, which is accumulated in the soil (no matter what the

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source is), should be used for plant biomass production and keeping the soil in the state of high fertility. Some authors (Akiyama et al., 2004; Barabasz and Vorisek, 2001) emphasize that often microbiological and biochemical processes occurring in soil environments outweigh the purely chemical reactions. The microorganisms within all the ecosystems are an indispensable biochemical factor. This biochemical factor is responsible for the totality of the transformation of biogens in the soil environment. The microorganisms also form the biological activity of biogens. How these transformations in the soil environment take place depends on many ecological and physiochemical elements (pH, soil capacity, content of macro and microelements, amount of organic carbon, the presence of xenobiotics etc.).

The first aim of this article was to evaluate how sewage sediments influence the phytosanitary potential of the soil environment. The second aim was to assess the activity of microorganisms which carry out the transformations of carbon and nitrogen compounds.

The experiment was conducted on agricultural land of the plant experimental station in Głubczyce. The experiment was carried out using the randomized block design method on the Cambisol. Used Cambisol are developed from silts clay. In the Ap layer, the content of the floated parts (< 0.02 mm diameter) was 50%, and the content of clay (< 0.002 mm) was 18%.

The whole experiment consisted of 3 objects, and 4 repetitions: C – soil without organic fertilization (as the control); S+SS – soil with a dose or sewage sludge 30 Mg·ha⁻¹ (7.2 Mg·ha⁻¹ d.m.); S+CM – soil with a dose of manure 30 Mg·ha⁻¹ (8.1 Mg·ha⁻¹ d.m.). Sewage sludge and manure were used once. Mineral fertilization and the appropriate agro-techniques were used according to the requirements of peas, as peas were cultivated on these fields. One marked “S” soil sample was collected from the whole field (during autumn, before start of experiment) on which the experiment was carried out. The results of the experiment are the arithmetic mean of the four repetitions.

Sewage sediments stored on stabilization pond from the Opole Wastewater Treatment Plant were used in the research project. Cattle manure from a farm in Głubczyce was also used in this study (properties of used materials presented in Table I).

The soil samples were collected during spring (May) from the Ap level of the experimental plots. The following analyses were done on the collected soil samples:

- pH in KCl;
- salinity based on conductivity of aqueous extracts (5:1) with the conductometric method;
- the content of macronutrients:
  - total nitrogen content with the method of Kjeldahl (PN-ISO-11261, 2002),
  - phosphorus content by PN-R-04023 DL (1996),
  - potassium content by PN-R-04022 DL (1996),
  - magnesium content by PN-R-04020 DL (2004);
- the content of micronutrients in 1M HCl by AAS method (Philips PU 9100X);
- the content of organic carbon by the Tiurin's method (PN-ISO-14235, 2003).

Statistic analyses (LSD) was carried out with the Statistica program.

Microbiological analyses included the designation of the number of ammonifiers on the liquid medium with peptone (PN C-04615-18), nitrifiers of phase I (NfI) and phase II (NfII) on Winogradsky medium (PN C-04615-20), bacteria fixing the non-symbiotically nitrogen. The nitrogen is from the following: Azotobacter sp. on the Fenglerowa medium (Pfeiffer-Maliszewski 1974), cellulolytic bacteria (Petrycka 1993), amylolitic microorganisms on the medium which contain starch (Pochon Tardieux 1962).

The examined groups of microorganisms were determined by inoculating the appropriate media with volumes in the range 0.1–1.0 ml coming from different tenfold serial dilutions. All different microorganisms were estimated by the standard dilution-plating procedure, with the exception of ammonifiers, nitrifiers of phase I and phase II and cellulolytic microorganisms, which were determined by the Most Probable Number (MPN). The MPN was calculated using McCrady’s Tables, for three parallel repeats. The general number of amylolitic bacteria and bacteria fixing nitrogen non-symbiotically from the Azotobacter sp., was shown in cfu/g·d.m. of soil. The culturing process of ammon-
The potential activity of the ammonification process (Dn), and the bonding of nitrogen was checked with Nessler’s reagent. The phase I of nitrification process was checked with Griess-Issolvay’s reagent to find out how active the process was. The activity of the phase II nitrification process was checked using diphenylamine. The color change was based on the reagents used and the modified Pochon method (Pfeiffer-Maliszewska 1974, Pochon Tardieux 1962).

All the readings were checked three times. The statistical calculations were subjected to variance analyses using Duncan’s test.

The organic materials used in the experiment were sewage sediments and manure. These materials had different hydration levels, and different levels of basic biogenic components. The analysis of the content of these components in the manure and sewage sediments showed that manure had more organic matter and more of the analyzed micro components (except calcium) and organic matter. The micro element quantitative analysis indicated a high content of calcium and phosphorous in the sewage sediment added to the soil. The amount of nitrogen, magnesium and potassium was average (Baran and Turski 1996, Mazur 1999). pH of the sewage sediment was neutral. The salinity of the soil reached 6.00 g KCl·kg⁻¹. The amount of organic matter in the manure was high (61.70%).

Organic fertilization in the form of sewage sediments or manure has a relevant impact not only on the chemical or physical properties of the soil but also on the biological properties (Grata and Krzyśko-Łupicka 2005, Joniec and Furlczak 2012, Pisarek 2007, Wolna-Murawka et al., 2007). Various changes can affect the number and activity of the microorganisms and can also affect the direction carried out by microbes. These changes include: those of pH of the soil, changes in the relations between nitrogen and carbon, changes in the quantity and quality of the organic matter, and the change in the sediment or manure doses (Fernandes et al., 2005, Kavadia et al., 2007, Wolna-Murawka et al., 2007). Some researchers (Singh and Agrawal 2008, Wong et al., 1998) showed that the sewage sediments can significantly lower the pH level of the fertilized soil by nitrification or by producing organic acid. After the external organic matter underwent transformation for 6 months, there was no change in the reaction of the soil but the amount of pH_KCl showed that all the analyzed soils from the experimental plots were slightly acidic (Table II).

The assessment of the assimilated content forms of phosphorus (based on the border number PN-R-04023 DL) showed that phosphorus content in the soil remained high such soil is considered I-st class. The added organic matter had no influence on the assimilated phosphorous. Those plots of soil which had been enriched with manure showed a 118 mg·kg⁻¹ increase of P. The assessment of the available potassium in the soil according to the border numbers (PN-R-04022 DL), indicated a high content of potassium (II-nd class) in all soils. The content of assimilated magnesium in the soils of the organically fertilized experimental plots showed (statistically unimportant) differentiation. The

<table>
<thead>
<tr>
<th>Objects</th>
<th>pH_KCl</th>
<th>Macronutrient (mg·kg⁻¹)</th>
<th>Micronutrient (mg·kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>K</td>
</tr>
<tr>
<td>S</td>
<td>6.4</td>
<td>94</td>
<td>226</td>
</tr>
<tr>
<td>S+SS</td>
<td>6.4</td>
<td>98</td>
<td>215</td>
</tr>
<tr>
<td>S+CM</td>
<td>6.4</td>
<td>118</td>
<td>278</td>
</tr>
<tr>
<td>C</td>
<td>6.4</td>
<td>94</td>
<td>216</td>
</tr>
<tr>
<td>LSD</td>
<td>n.s.</td>
<td>n.s.</td>
<td>49</td>
</tr>
</tbody>
</table>

n.s. – not significant
soils were thus divided into two categories: soils with an average abundance (S, CM) and soils with a low abundance (S+SS, C) (PN-R-04020 DL). The number of the assimilated forms of the analyzed microelements (Mn, Cu, Zn, Fe) indicated they occur in average amounts. The experiment confirmed the significant influence of the injected sewage sediments on the level of manganese in the experimental soils.

Following the enrichment of the soil with sewage sediment and manure, there was a statistically significant increase in the content of nitrogen and organic carbon noted in some of the experimental plots (Table III). On object C, the increase of carbon and nitrogen was statistically non-significant and probably associated with microbes and with the transformation of the secretions of the cultivated plants (*Pisum sativum*). MAAs it results from many researchers (Colnaght *et al.*, 1997, Wanic and Nowicki 2000) have shown that cultivation of fabaceae significantly affects the biologic life of the soil, the biochemical processes within the soil, and the sanitary state of the soil.

The value of the C:N ratio in the soils show the content of humus in the nitrogen. The organic sewage sediment and manure added to the soil had a wider range of C:N (13.9 for SS; 15.8 for CM). According to Smith (1996), this wide range in the sewage sediment and manure, points to an average and low susceptibility to mineralization processes. As far as the speed and type of the processes are concerned, Griffin *et al.* (2002) and Smith (1996) assumes that nitrogen in manure can be temporarily fixated during immobilization.

The analysis of the starting material shows that the number of ammonificators in the soil, in the sediment, and in the manure was comparable. However the intensity of this process was much higher in the manure. The manure had a smaller amount of phase I nitrifiers and the intensity of the nitrification process was also lower. However, in the manure and in the sediment, the number of phase II nitrifiers and the intensity of the nitrification process was similar but much higher than in the non-fertilized soil (Fig. 1, 2).

Sewage sediments and manure, due to the considerable amount of nitrogen they have, the presence of organic matter, and their fertilization use, can influence the microbiologic transformation of nitrogen soil, and

<table>
<thead>
<tr>
<th>Objects</th>
<th>C</th>
<th>N</th>
<th>C:N</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>9.80</td>
<td>1.07</td>
<td>9.1</td>
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<tr>
<td>SS</td>
<td>163.90</td>
<td>11.8</td>
<td>13.9</td>
</tr>
<tr>
<td>CM</td>
<td>356.10</td>
<td>22.5</td>
<td>15.8</td>
</tr>
<tr>
<td>S+SS</td>
<td>14.50</td>
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</tr>
<tr>
<td>S+CM</td>
<td>15.20</td>
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</tr>
<tr>
<td>C</td>
<td>11.70</td>
<td>1.19</td>
<td>9.8</td>
</tr>
</tbody>
</table>

LSD (between objects with soil) 4.6 0.31 0.88

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Fig. 1. Number of different groups microorganisms in the starting material expressed in MPN/ g d.m. (ammonificators, nitrifiers) and in cfu/g d.m (*Azotobacter sp.*)

Fig. 2. Potential activity of different groups microorganisms in the starting material
can influence the number of ammonificators, nitrifiers microorganisms or the microorganisms which fixate the nitrogen (Fernandes et al., 2005, Grata and Krzyśko-Łupicka 2005, Piontek and Lonc 2000). The nitrogen present in the organic substance is transformed in the process of ammoniafication to the form NH$_4^+$. This form is collected by the plants or easily undergoes changed or unchanged sorption and the NH$_4^+$ also remains in the soil solution. The NH$_4^+$, however, is not stable. As a result of nitrogen assimilation together with the microorganisms from the “Nitroso” group, the NH$_4^+$ undergoes oxidation and first becomes nitrites (NO$_2^-$) and then becomes nitrates (NO$_3^-$). This is why finding out the amount and the activity of the mentioned microorganisms in the soil is so important (Sorensoen 2001).

Fertilizing with sewage sediment and manure slightly increased the amount of ammonificators (about 19%) compared to non-fertilized soil (Fig. 3). The amount of these microorganisms was about 1.11–1.39 × 10$^8$ cfu/g d.m. Fertilizing with only manure led to the intensification of the ammonification process compared to non-fertilized soil and soil fertilized with just sediment.

Our research also shows, that the sewage sediment stimulated the development of the nitrifiers of phase I in the soil. The manure stimulated the development of nitrifiers of phase II (Fig. 3). After adding the manure and the sewage sediment to the soil, the nitrification process of phase I was noted to be significantly higher in intensity but there was an impairment of the phase II (Fig. 4). Jezierska-Tys et al. (2004) noted the sediment had a stimulating impact on the amount of the nitrifiers but simultaneously a inhibitory impact on the intensity of the processes carried out by the nitrifiers. If there is an excess of organic matter, a decrease in the speed and in the efficiency of the nitrification can take place without any changes in the amount of the nitrifiers as the nitrifiers contains a lot of N-NH$_4^+$. Such a large amount can inhibit this process (Lopez-Valdez et al., 2010; Strzelec and Kobus, 1997). In an environment containing readily soluble organic substances, the nitrifiers bacteria can survive in an inactive state (Strzelec and Kobus, 1997). The intense development of the nitrifiers could be caused by the activity of the ammonificators. The reason is, that this development is the strongest in a slightly alkaline reaction and an environment which is rich with ammonium nitrogen (Johanson et al., 1999; Lopez-Valdez et al., 2010). When comparing the processes of ammonification and nitrification, both the amount and the intensity of the ammonification process were found to be higher than the amount of the nitrifiers in both phases and the intensity of the nitrification process. A smaller amount of the nitrifiers in relation to the ammonificators is beneficial for the of environment. The nitrate nitrogen formed in the process of nitrification is a less stable compound than ammonium nitrogen. The nitrate nitrogen can easily be washed out from the soil and find its way into ground water and surface water. Plants can also
accumulate nitrate nitrogen which could cause a threat to both humans and animals (Kobus 1996).

Our results did not show a significant relationship between the amount of the ammonicators and nitrifiers, and the intensity of both processes. Jezierska-Tys et al. (2004), and Szostak et al. (2005) also did not notice a dependence between the amount of the discussed microorganisms and the intensity of the processes which the microorganisms undertake.

The quantitative and qualitative composition of the organic matter, the value of the C:N ratio, the level of sorption, the content of heavy metals, and the content of ammonium nitrogen can significantly affect the microorganisms fixating the atmospheric N (Grata and Krzyśko-Łupicka 2008, Martensson and Torstensson 1996). These kinds of impacts involve the inhibition of the nitrogenase activity, the synthesis of the nitorgenase (inhibition of the formation of the glutamine synthetase), and the reduction in the diazotroph population (Colnaghi et al., 1997; Kavadia et al., 2007; Tsagou et al., 2003).

The research on the microorganisms fixating N, non-symbiotically, showed that the amount of Azotobacter sp. was significantly higher in the soil (S) \(4,0 \times 10^4\) cfu/g d.m and in the manure (CM) \(5,0 \times 10^4\) cfu/g d.m, than in the sediment (SS) \(1,66 \times 10^4\) cfu/g d.m. It is important to note, that an intense fixation process of N \(_2\) took place only in the manure (CM) (Fig. 1, 2). After applying the manure, there was a significantly higher amount of Azotobacter sp. in the soil (S+CM) \(12,0 \times 10^4\) cfu/g d.m compared to the soil fertilized with sewage sediment (S+SS) \(6,66 \times 10^4\) cfu/g d.m and compared to non-fertilized soil (C) \(3,1 \times 10^4\) cfu/g d.m (Fig. 3). However, the fixation process of N \(_2\) took place with the same intensity in all the variants, with only a slight, not statistically significant predominance in the soil which had been enriched with manure (S+CM) (Fig. 4). The analysis of cellulolytic microbes showed more of these microbes in the manure (CM) and in the sediment (SS) \(1,18 \times 10^4\) cfu/g d.m, than in the soil (S) \(5,49 \times 10^4\) cfu/g d.m. After fertilizing, the soil with sediment (S+SS) \(2,35 \times 10^4\) cfu/g d.m showed the most microbes. The soil with manure (S+CM) \(5,44 \times 10^4\) cfu/d.s.m showed the least amount of microbes, as can be seen in Fig. 5.

The number and activity of this group of microorganisms may be related to content of organic matter available, the dose of fertilizer, while the coefficient of transformation of carbohydrates in the soil depends on the structure of the soil, pH, and degree of contamination of the environment. Therefore, results obtained by various investigators are differ. There was a fluctuations or an increase in their numbers, especially after high doses of organic matter (Mazur 1999, Wolna-Murawka et al., 2007).

The analysis of the amylolitic microorganisms amount showed that manure (CM) and the sediment (SS) had 10–100 times more microorganisms than the soil (S) \(2,9 \times 10^4\) cfu/g d.m. Manure had more amylolitic microorganisms and sediment had less. Adding organic matter to the soil in the form of manure and sewage sediment, resulted in a 10 fold increase in the amount of this group of microbes, especially in the S+CM \(5,33 \times 10^4\) cfu/g d.m) combination, compared to non-fertilized soil (C) \(4,3 \times 10^4\) cfu/g d.m) (Fig. 5).

**Conclusion.** Microorganisms and microfauna together with the vegetation are considered to be the factors which determine the direction of the biochemical and chemical changes occurring in the soil environment. The microorganisms and microfauna also form the biological activity of cultivated soils and models the physicochemical properties of the soil (Dumontet et al., 2001, Kornillowicz-Kowalska and Bohacz 2002, Stuczyński et al., 2003, Wiegand et al., 2004). The microorganisms which have a metabolism ability to transform organic compounds play a significant role in the previously mentioned processes. The organic materials added to the soil are mainly used by the microorganisms as a source of carbon. Later, the microorganisms use the added organic materials injected in the soils (after the initial decomposition) as a source of nitrogen and other components. In the first stage of the transformation process of organic matter, a growth in the general number of microorganisms takes place. In the following phases, the presence of specialized strains of microbes is...
Table IV
Correlation coefficients between some parameters

<table>
<thead>
<tr>
<th>Feature (X)</th>
<th>Feature (Y)</th>
<th>rk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonificators</td>
<td>P</td>
<td>0.923</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>0.975</td>
</tr>
<tr>
<td></td>
<td>Fe</td>
<td>0.610</td>
</tr>
<tr>
<td></td>
<td>Mn</td>
<td>0.912</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>0.612</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td>-0.911</td>
</tr>
<tr>
<td>Nitrifiers I phase</td>
<td>P</td>
<td>0.812</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>0.601</td>
</tr>
<tr>
<td></td>
<td>Fe</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Mn</td>
<td>0.881</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td>-0.871</td>
</tr>
<tr>
<td>Nitrifiers II phase</td>
<td>P</td>
<td>-0.870</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>-0.688</td>
</tr>
<tr>
<td></td>
<td>Fe</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Mn</td>
<td>-0.923</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td>-0.921</td>
</tr>
<tr>
<td>Azotobacter sp.</td>
<td>P</td>
<td>0.987</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>0.961</td>
</tr>
<tr>
<td></td>
<td>Fe</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Mn</td>
<td>0.945</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td>-0.991</td>
</tr>
</tbody>
</table>

n = 9
significantly p = 0.001 (above 0.783)
significantly p = 0.05 (above 0.600)

nitrates of phase I and II, and the Azotobacter sp. showed differentiation. Such variation depended on the properties of the chemical surface (see Table III). The potential activity was shown by the significant value of the correlation coefficient (Table IV).

The presence of forms of copper on the surface were found to decreases the biological activity of the analyzed groups of microbes. An increase in the activity of ammonificators, nitrifies of phase I, and Azotobacter sp. contributes to the presence of P, K, Fe, Mn and Zn in the soil. The activity of the nitrifies of phase II is formed differently. The nitrifies of phase II show a reduced activity in the presence of the analyzed macro- and microelements.

Literature


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Polish Committee for Standarization, 1996. Soil quality. PN-R-04022 DL.

Polish Committee for Standarization, 2004. Soil quality. PN-R-04020 DL.


**Fast Identification of Yersinia pestis, Bacillus anthracis and Francisella tularensis Based on Conventional PCR**

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**Abstract**

Rapid and accurate diagnostic tools for detection and identification of *Y. pestis*, *B. anthracis* and *F. tularensis* are essential for timely initial appropriate treatment of exposed individuals, which will be critical to their survival, as well as for reduction of the public health impact and the spread of the disease. The paper presents application of fast polymerases and fast dry electrophoresis in conventional PCR as an alternative for real-time PCR application for detection and identification of the above pathogens. The proposed method takes less than 50 min. to obtain final results of the tests and is cheaper than real-time PCR.

**Key words:** *B. anthracis, F. tularensis, Y. pestis*, detection, PCR

*Yersinia pestis*, *Bacillus anthracis* and *Francisella tularensis* are etiological agents of plague, anthrax and tularemia, respectively – severe diseases in humans and animals. Although rare in majority of developed countries, the three pathogens still exist in nature, especially in endemic areas, and cause diseases. For example, many parts of Europe, Asia, Africa, Australia, and North, Central, and South America are regarded as anthrax endemic areas (Gasper and Watson, 2001). Plague is endemic in many natural foci of Asia, Africa, and the Americas (Riehm et al., 2011). Whereas the most virulent *F. tularensis* subspecies are found mainly in North America (Vogler et al., 2009). Development of mass tourism to endemic regions of diseases caused by *Y. pestis*, *B. anthracis* and *F. tularensis* can result in transmission of the diseases to countries regarded as free from these pathogens. Moreover, the pathogens are listed as a category A bio-threat agents according to the Centers for Disease Control and Prevention (CDC) of USA. The category A agent is an organism that poses a risk to national security because it can be easily disseminated or transmitted from person to person, results in high mortality rates, has the potential for public health impact, might cause public panic and social disruption and requires special action for public health preparedness (http://emergency.cdc.gov/agent/agentlist-category.asp).

Rapid and accurate diagnostic tools for detection of these pathogens are essential for timely initial appropriate treatment of exposed individuals which will be critical to their survival as well as for reduction of the public health impact and the spread of the disease. PCR has been regarded as an accurate diagnostic tool for detection and identification of *Y. pestis*, *B. anthracis* and *F. tularensis*. Application of real-time PCR technology has made this tool very rapid. However, the real-time PCR equipment is not always accessible in local laboratories in many countries, especially in endemic areas which are often located in the developing countries.

In this paper we present a possibility of significant time reduction necessary for *Y. pestis*, *B. anthracis* and *F. tularensis* identification by use of rapid polymerases and rapid dry electrophoresis in conventional PCR assay.

In this study five fast polymerases were tested: Phire Hot Start II DNA Polymerase (Finnzymes), PyroStart Fast PCR Master Mix (Fermentas), AmpliTaq Gold Fast PCR Master Mix (Applied Biosystems), SapphireAmp Fast PCR Master Mix (Takara), Qiagen Fast Cycling PCR (Qiagen). PCR conditions were followed according manufacturers’ instructions (Table I). The primers for species and subspecies identification were used as described earlier for *Y. pestis* by Zhou D et al. (2004), for *B. anthracis* by Jackson PJ et al. (1998), for *F. tularensis* by Johansson A et al. (2000), Tomaso H et al. (2007) and Barns SM et al. (2005). DNA samples isolated from the following strains were used: *B. anthracis* BL1 and BL6, *Y. pestis* NCTC00570 and 03–1506, *F. tularensis* spp. *tularensis* Schu S4, *F. tularensis* spp. *holarctica* A104-15,

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**SHORT COMMUNICATION**
$F. \text{tularensis}$ spp. novicida Ft26. The reactions were conducted comparatively in Eppendorf Mastercycler and in Bio-Rad C1000 Thermal Cycler. Dry electrophoresis of the products was conducted using E-Gel Base System (Invitrogen). The maximal time of electrophoresis was 10 minutes.

All the tested polymerases amplified the genetic markers in sizes ranging from 136 bp to 1200 bp efficiently (as an example results for $F. \text{tularensis}$ are presented in Fig. 1). The duration of 30 cycles of PCR varied from 63 min to 72 min on Eppendorff Mastercycler and from 36 min to 47 min on Bio-Rad C1000 Thermal Cycler (Table I). As it was supposed based on the theoretically calculated minimal duration of thermocycling the fastest polymerase was SapphireAmp Fast PCR Master Mix (Takara). The blue dyes and a material for increasing the specific gravity of the solution contained in the master mix did not influence the electrophoresis results although the manufacturer of the E-Gel base system warns that loading buffer with tracking dye may mask DNA bands. Almost as fast as SapphireAmp Fast PCR Master Mix was Phire Hot Start II DNA Polymerase (Finnzymes). Preparation of a reaction mixture with Phire Hot Start II DNA Polymerase takes a little more time as it was the only polymerase tested that was not available as “ready to use” mix containing all necessary reagents except primers. The disadvantage of PyroStart Fast PCR Master Mix was that the DNA bands were getting blurred during dry electrophoresis. It was probably caused by high concentration of salts in the PCR buffer. This effect can be overcome by dilution of the PCR products before loading onto the gel.

Speed of thermocycling reaction depends not only on the amount of time spent for incubation at each temperature step but also from the time taken to reach the incubation temperature (the ramp time) what is a characteristic of a PCR device. Comparison of two thermocyclers conducted in these studies revealed possibility of up to 43% of reduction of PCR duration depending on the thermocycler used. Matero et al. (2011) also revealed significant differences in duration of thermocycling comparing two real-time PCR instruments. As real-time PCR is regarded as faster than conventional PCR, we reviewed literature to compare real-time PCR duration with different instruments used for $B. \text{anthracis}$ detection. The comparison concerned only duration

<table>
<thead>
<tr>
<th>PCR conditions applied for tested polymerases and duration of thermocycling.</th>
</tr>
</thead>
</table>
| Phare Hot Start II DNA Poly.
(Finnzymes) | PyroStart Fast PCR Master Mix (Fermentas) | AmpliTaq Gold Fast PCR Master Mix (Applied Biosystems) | Sapphire Amp Fast PCR Master Mix (Takara) | Qiagen Fast Cycling PCR (Qiagen) |
| Initial denaturation | 30 s | 1 min | 10 min | 1 min | 5 min |
| Denaturation | 5 s | 1 s | 3 s | 5 s |
| Anealing | 5 s | 5 s | 3 s | 5 s |
| Extension | 10 s | 25 s | 15 s | 10 s | 5 s |
| Final extension | 1 min | 10 s | 10 s | – | 1 min |
| Theoretical minimal time of 30 cycles | 11 min 30 s | 16 min 40 s | 20 min 40 s | 11 min | 21 min |
| Thermocycling duration with Bio-Rad C1000 Thermal Cycler | 39 min | 42 min | 47 min | 36 min | 47 min |
| Thermocycling duration with Eppendorf Mastercycler | 63 min | 66 min | 72 min | 65 min | 72 min |

![Fig. 1. PCR products obtained for $F. \text{tularensis}$ spp. novicida (A) and $F. \text{tularensis}$ spp. holarctica (B) after 7 minutes of dry electrophoresis. Lines: 1 and 6–16S rRNA marker (1200 bp), 2 and 7 – tul marker (428 bp), 3 and 8 – FtC marker (170 bp), 4 and 9 – pdpD marker (285 bp in $F. \text{tularensis}$ spp. novicida, negative in $F. \text{tularensis}$ spp. holarctica), 5 – E-Gel 1 kb Plus DNA Ladder.](image-url)
of thermocycling, excluding time of DNA extraction. The comparison (Table II) allowed to draw a conclusion that application of fast polymerases and rapid dry elecrophoresis in conventional PCR enables to obtain results in time similar as in real-rime PCR. The minimal time necessary for obtaining results of identification of the pathogens was less than 50 min. It is worth to underline that use of conventional PCR with fast enzymes and rapid dry elecrophoresis is cheaper than use of real-time PCR in terms of equipment as well as reagents, what means that it could be more accessible to local laboratories. Our calculation revealed that the costs of analysis of one sample using fast conventional PCR and dry elecrophoresis varied from 0.95 euro to 3.15 euro depending on the fast polymerase used, whereas costs of analysis of one sample using real-time PCR are 2.50–12 euro. Even more significant are differences in costs of equipment. The costs of a conventional thermocycler together with equipment for dry elecrophoresis are 3900–7450 euro. Moreover, the fast PCR assay together with rapid dry elecrophoresis might be used in the field as the instruments are small, light and portable.

Acknowledgment
The studies have arisen from the Project QUANDHIP No 20102102 which has received funding from the European Commission in the framework of the Health Programme.

Literature


<table>
<thead>
<tr>
<th>Detected markers</th>
<th>Instrument (manufacturer)</th>
<th>Thermocycling duration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pagA, capB</td>
<td>ABI 7300/7500 (Applied Biosystems)</td>
<td>100 min</td>
<td>(Matero et al., 2011)</td>
</tr>
<tr>
<td>pagA, capB</td>
<td>RAZOR (Idaho Technology Inc.)</td>
<td>40 min</td>
<td>(Matero et al., 2011)</td>
</tr>
<tr>
<td>plcR, gyrA</td>
<td>LightCycler 480 (Roche Diagnostics)</td>
<td>60 min</td>
<td>(Derzelle et al., 2011)</td>
</tr>
<tr>
<td>pagA, capB</td>
<td>LightCycler (Roche Applied Science)</td>
<td>60 min</td>
<td>(Bell et al., 2002)</td>
</tr>
<tr>
<td>pagA</td>
<td>Smart Cycler (Cepheid)</td>
<td>55 min</td>
<td>(Selvapandiyan et al., 2005)</td>
</tr>
<tr>
<td>pagA, capB</td>
<td>Rotor-Gene 6000 (Corbett Life Science)</td>
<td>52 min</td>
<td>(unpublished data)</td>
</tr>
</tbody>
</table>

Rhizobia are ubiquitous soil microorganisms but the diversity and density of soil populations of these bacteria depend on soil properties, crop rotation, agricultural practices and also to the great extent on the presence of wild species of leguminous plants in a given area. In regions or countries, like Poland, where soybean is not an indigenous plant and where this crop is not grown frequently soils are usually void or deficient in rhizobia nodulating soybean (Bushan, 1998; Sadowsky and Graham, 1998; Prevost and Bromfield, 2003, Martyniuk et al., 2005; Cheminingwa and Vessey, 2006). Under such conditions soil inoculation or pre-sowing seed pelleting of soybean seeds with inoculants containing root-nodule bacteria specific for soybean result in a significant increase of nodulation and seed yields of this crop (Thies et al., 1991; Singleton et al., 1992; Bushan, 1998; Graham and Vance, 2003). Moreover, it has been shown under gnotobiotic conditions and in soil-less pot experiments that mixed inoculants containing rhizobia and other beneficial bacteria, like Azotobacter spp., were more effective than inoculants consisting of rhizobia alone in stimulation of nodulation, nitrogen fixation and in consequence the yields of legumes were higher (Burns et al., 1981; El-Bahrawy, 1983; Rodelas et al., 1999).

The objective of this study was to determine, in pot and micro-plot experiments with natural soil, whether nodulation and seed yields of soybean are influenced by pre-sowing seed inoculation with Bradyrhizobium japonicum alone or with mixed inoculants containing soybean rhizobia and Azotobacter chroococcum and to compare obtained results.

The Culture Collection of $\text{N}_2$-fixing Bacteria belonging to Department of Agricultural Microbiology of the Institute of Soil Sc. and Plant Cultivation in Pulawy was the source of all the bacteria used in this work. All Bradyrhizobium japonicum strains were isolated from soybean nodules and they originated from: Poland (strains: 78B, L, PO and PR), USA (strains 138, 110 and 94P), Australia (strain CB82), North Korea (strain KR) and from Sweden (strain II). Stock cultures of rhizobia were maintained at 4°C on slants of yeast extract mannitol agar (YEMA) supplemented with 3 g CaCO$_3$ L$^{-1}$ (Vincent, 1970). These bacteria were used to inoculate soybean seeds in a pot experiment. Viable cell numbers (colony forming units – c.f.u.) of the rhizobia in liquid cultures and on inoculated soybean seeds were counted by standard dilution plate procedures on Congo red-YEMA (Vincent, 1970, Martyniuk et al., 2005). Azotobacter chroococcum strain 17/08 was isolated from
fresh soil collected from an experimental field at the Experimental Station belonging to the Institute of Soil Science and Plant Cultivation (ISSPC) in Puławy, using plates with modified Burk’s N-free agar medium inoculated with serial dilutions of this soil in sterile water (Martyniuk and Martyniuk, 2002). This medium was also used to maintain stock cultures of *A. chroococcum* 17/08 and to count numbers of c.f.u. of these bacteria in liquid cultures and on soybean seeds.

Finely milled brown coal, mixed with 1% of CaCO₃, to adjust the pH to 6.6–6.8, was used as the bacterial carrier in all inoculants. The carrier material was obtained from a commercial producer of rhizobial inoculants in Walcz, Poland. Portions of moistened (5%) carrier weighing 200g were placed in polypropylene bags and sterilized by autoclaving in 121°C for 60 min. Batches of 50 ml of YEML medium and N-free Burk’s medium were used to culture the rhizobia and *A. chroococcum* 17/08, respectively, in 250-ml Erlenmeyer flasks on a shaker platform rotating at 100 rpm. Cultures of the bacteria in the late log-phase of growth with approximately 2–5×10⁸ c.f.u. ml⁻¹ were used to inoculate bags containing sterile carrier. To prepare mixed inoculants bags with sterile carrier were inoculated with 40 ml of broth cultures of particular rhizobial strain and 40 ml broth culture of *A. chroococcum* 17/08, respectively, in 250-ml Erlenmeyer flasks on a shaker platform rotating at 100 rpm. Cultures of the bacteria in the late log-phase of growth with approximately 2–5×10⁸ c.f.u. ml⁻¹ were used to inoculate bags containing sterile carrier. To prepare mixed inoculants bags with sterile carrier were inoculated with 40 ml of broth cultures of particular rhizobial strains and 40 ml broth culture of *A. chroococcum*. In the case of inoculants containing the rhizobia alone 40 ml of Burk’s liquid medium was added instead of *A. chroococcum* culture. The inoculants were incubated for 24 hours at room temperature and then used to inoculate soybean seeds at the rate of 2 g per 100 g of seeds.

Polish cultivar “Aldana” of soybean [*Glycine max* (L) Merrill] was grown in a pot experiment (2010) and in two micro-plot experiments under field conditions conducted in 2011 and 2012. In the pot experiment, Mitscherlich pots filled with 7 kg of the soil collected from the experimental field at the ISSPC Experimental Station in Puławy were used. This soil (sandy loam) had the following basic characteristics: pH 6.2, 1% org. C, 60% sand, 31% silt and 9% clay. The soil contained low levels of P (about 2 mg kg⁻¹), K (about 100 mg kg⁻¹), Mg (about 40 mg kg⁻¹), Ca (about 600 mg kg⁻¹) and soil moisture adjusted to 60% of WHC (water holding capacity). This soil moisture level was maintained throughout the entire experimental period.

At the flowering stage of soybean plants, one pot from each treatment was removed to examine root systems for nodulation intensity according to the following scale: 0 – no nodules on the roots, 1 – single nodules on lateral roots only, 2 – small clusters of nodules on the tap root and single nodules on the laterals, 3 – large clusters of nodules on the tap root and numerous nodules on the laterals. At physiological maturity of the soybean plants the experiment was terminated to determine seed yields and some yield components.

Micro-plot experiments, arranged in a randomized split-plot design, were carried out during the 2011 and 2012 growing seasons on a field at the ISSPC Experi-
mental Station in Pulawy. The experiments included the following treatments, each consisting of four replicated plots: I – soybean seeds (cv. Aldana) treated with inoculant containing *Bradyrhizobium japonicum* strain 94P, II – soybean seeds treated with mixed inoculant of strain 94P and *A. chroococcum* 17/08 and III – uninoculated seeds. About 24 hours before inoculation soybean seeds were treated with commercial chemical seed dressing “Sarfun”. Plants were grown on 1 m² plots consisting of 3 rows spaced 33 cm apart. Soil fertilization and other agro-technical practices followed general recommendations for cultivation of soybean.

At the flowering stage of soybean development, five plants from each replicated plots were dug out to assess root systems for nodulation intensity according to the scale used in the pot experiment and to count the total number of bacteria from the genus *Azotobacter* in non-rhizosphere soil samples and in the rhizosphere soil closely adhering to soybean roots. At the physiological maturity stage all soybean plants from the central rows were collected to determine seed yields and some yield components. All data were subjected to the analysis of variance using Anova test.

In the pot experiment, all inoculants containing either *Bradyrhizobium japonicum* strains alone or mixtures of the rhizobial strains with *Azotobacter chroococcum* strains 17/08 significantly increased nodulation rate, pod numbers and seed yields per plant of soybean as compared to the untreated control plants (Table I). Of the tested *B. japonicum* strains only two of them (110 and PO) had significantly lower ability to stimulate nodulation of soybean than other strains of *B. japonicum*, but with respect to pod numbers and soybean seed yields these differences were less pronounced. The inoculants containing mixed cultures of *B. japonicum* strains and *A. chroococcum* were generally similar in their effectiveness in increasing nodulation rate of soybean plants with the exception of the strain PO which was less effective when used as pure culture (Table I). Strain 94P of *B. japonicum* both applied as pure culture or in mixture with *A. chroococcum* 17/08 gave relatively the highest soybean seed yields in the pot experiment and this strain has been chosen for further studies under field conditions (microplot experiments).

In the micro-plot experiments conducted in 2011 and 2012 pre-sowing inoculation of soybean seeds with the inoculant containing *B. japonicum* strain 94P alone and with the mixed inoculant containing the strain 94P and *A. chroococcum* 17/08 resulted in a significant increase of nodulation intensity, pod numbers and seed yields of soybean, as compared to the uninoculated control (Table II).

Table II

<table>
<thead>
<tr>
<th>Seeds inoculated with:</th>
<th>Counts of <em>A. chroococcum</em> c.f.u g soil⁻¹</th>
<th>Nodulation rating</th>
<th>Number of pods plant⁻¹</th>
<th>Seed weight g square m⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, no inoculation</td>
<td>35 a*</td>
<td>0.4 a</td>
<td>8.7 a</td>
<td>172 a</td>
</tr>
<tr>
<td><em>B. japonicum</em> 94P</td>
<td>28 a</td>
<td>2.1 b</td>
<td>12.2 b</td>
<td>286 b</td>
</tr>
<tr>
<td><em>B.j.</em> 94P + <em>Azotobacter</em></td>
<td>61 b</td>
<td>2.1 b</td>
<td>13.1 b</td>
<td>292 b</td>
</tr>
</tbody>
</table>

* Values in columns followed by the same letter are not significantly different (α = 0.05)

Previous studies with different legumes have clearly shown that seed inoculation with symbiotic root-nodule bacteria is very effective in improving nodulation and yields of these crops when they are grown on soils deficient in infective strains of rhizobia or on soils containing low numbers of these bacteria (Thies et al., 1991; Singleton et al., 1992; Bushan, 1998; Graham and Vance, 2003). This was the case with the soil used in our experiments. This soil contained only about 12 cells g⁻¹ of rhizobia nodulating soybean and thus pre-sowing seed inoculation with the tested inoculants resulted in markedly higher nodulation rates and soybean seed yields as compared to those obtained in the control treatment without seed inoculation (Table II).

Results shown in Table II, which are means for two growing seasons (2011 and 2012), indicate that the mixed inoculants containing the rhizobial strain 94P and *A. chroococcum* strain 17/08 were not superior in comparison to the inoculant containing *B. japonicum* 94P alone.

In pot experiments conducted under gnotobiotic conditions or with the use of sterile soils (Burns et al., 1981; El-Bahrawy, 1983; Rodelas et al., 1999) it has been shown that nodulation intensity, efficiency of nitrogen fixation and yields of some legumes inoculated with mixed cultures of rhizobia and other beneficial microorganisms, like *Azotobacter* spp. and *Azospirillum* spp., were higher as compared to those inoculated with the rhizobia alone. Results of our pot and micro-plot experiments with unsterile soil naturally...
colonized with various soil microorganisms indicate that addition *Azotobacter chroococcum* strain 17/08 to the inoculant did not improve symbiotic interaction between *Bradyrhizobium japonicum* 94P and soybean plants (Table II).

The prerequisite to obtain positive effects of seed or soil inoculation with beneficial microorganisms on the plant growth is proliferation of microorganisms on roots or in the rhizosphere soil (Bashan, 1998; Rodelas et al., 1999). To find out if *A. chroococcum* introduced onto soybean seeds proliferated in the rhizosphere soil of this crop we counted total numbers of *Azotobacter* spp. cells in non-rhizosphere soil and in soil closely adhering to the roots of soybean inoculated with the tested inoculants. The results presented in Table II show that the number of *Azotobacter* spp. in the rhizosphere soils of soybean treated with the inoculant containing *A. chroococcum* 17/08 remained low (61 c.f.u. g soil$^{-1}$), even though it was significantly higher than that in the rhizosphere soil of soybean treated with *B. japonicum* 94P alone and in the non-rhizosphere soil. Competitive interactions with other soil microorganisms and at slightly acid pH 6.2 of the soil used in our studies were probably the main reasons for the limited proliferation of *A. chroococcum* in the rhizosphere soil of soybean. Bacteria of the genus *Azotobacter* prefer neutral soils in which their numbers range from several hundreds to $10^4$ c.f.u. in g of soil (Bashan, 1998; Rodelas et al., 1999; Martyniuk and Martyniuk, 2002).

**Acknowledgements**

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**Literature**


There are several groups of pathogenic *Escherichia coli* (*E. coli*) strains associated with gastrointestinal infections in humans. Enteropathogenic *E. coli* (EPEC) in contrast to other pathogenic *E. coli* characterize localized adherence pattern (LA) in which compact clusters of bacteria adhere to epithelial cells. The LA pattern results from specific to EPEC pathotype virulence factors involved in adhesion i.e. bundle forming pili (BFP) that initiate binding of bacteria to the host cells. The *bfp* operon encoding BFP fimbriae is localized on a 50–70 kDa plasmid called EPEC Adherence Factor (EAF). The binding of EPEC to host cells is crucial to the delivery of effector molecules secreted into the enterocytes via a type III secretion system (TTSS) that cause cytoskeleton rearrangement and actin accumulation beneath clusters of adhering bacteria. This specific lesion called ‘attaching and effacing’ (AE) characterize loss of microvilli and intimate attachment of EPEC to the epithelial cells of the small intestine (Clarke et al., 2003). All genes required to produce AE lesions are localized in EPEC on a large chromosomal pathogenicity island, locus of enterocyte effacement (LEE). The LEE region contains the *eae* operon encoding the outer membrane protein intimin that interacts with translocated intimin receptor Tir delivered into the host cell membrane via TTSS. The *eaeA* and *bfpA*-positive EPEC strains that carry genes involved in LA adherence and produce AE lesions are classified as typical (tEPEC). Unlike tEPEC, the *eaeA*-positive but *bfpA*-negative EPEC are classified as atypical (aEPEC) and display LA-like (LAL), diffuse adherence (DA) or aggregative adherence (AA) patterns (Clarke et al., 2003).

Enteropathogenic *Escherichia coli* strains (EPEC) carrying the *eae* gene encoding intimin are divided into typical strains producing bundle forming pili, encoded by the *bfpA* gene, and atypical strains lacking the gene. In the study typical and atypical EPEC that did not agglutinated with EPEC polyvalent antisera but carrying virulence factors characteristic to other pathogenic *E. coli* i.e. diffusely adhering and enteroaggregative *E. coli* were isolated from 24 (43.6%) of 55 children > 10 years old with persistent diarrhea. These results indicated that non-typeable typical and atypical EPEC can contribute to chronic intestinal infections in teenagers.

**Key words:** adherence patterns, typical and atypical enteropathogenic *E. coli*
References

The remaining 17 (29.2%) adhered to the epithelial cells in LA pattern. (Table I). All PCR amplifications were performed in a DNA-Engine PT200 thermal cycler (MJ Research Waltham, MA, USA).

Table I

Primer sequences and PCR conditions used in the study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR primers</th>
<th>PCR Product size (bp)</th>
<th>References conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>eaeA</td>
<td>(F) CACAGAATATAACTGACTAAAATG (R) AAAAACGCTGACCCGGACCTTAAT</td>
<td>55°C 20 s, 30 cycles</td>
<td>376 Lindqvist, 1997</td>
</tr>
<tr>
<td>bfpA</td>
<td>(F) AGTGGTGCTTGGGTTGCTGC (R) GGCCCTTATCCAAACCTGTGA</td>
<td>69°C 1 min, 30 cycles</td>
<td>326 Gunzburg et al., 1995</td>
</tr>
<tr>
<td>hlyA</td>
<td>(F) GCTTGCAAGAACATCGGCTGCAAATGA (R) CTGTTGACCAGTTGTTGATTAG</td>
<td>58°C 1 min, 30 cycles</td>
<td>561 Müller et al., 2009</td>
</tr>
<tr>
<td>afaD</td>
<td>(F) GTCAGGTCGCCGATATCAGT (R) CACTCTCCCTGTGAACTTCA</td>
<td>65°C 30 s, 35 cycles</td>
<td>250 Sobieszczanska et al., 2012</td>
</tr>
<tr>
<td>daaD</td>
<td>(F) GGGATATAAGGAGATGATGCG (R) TATTCCTGTGACACACACA</td>
<td>60°C 30 s, 35 cycles</td>
<td>437 this study</td>
</tr>
<tr>
<td>aggB</td>
<td>(F) GCATATTACCGATGTCCTGCG (R) CCTCTTGTATCTAGACATTCA</td>
<td>58°C 30 s, 30 cycles</td>
<td>421 Sobieszczanska et al., 2012</td>
</tr>
<tr>
<td>aggR</td>
<td>(F) CTATATTGCAACATCGATTGA (R) ATGAAGTATACATCTGATAT</td>
<td>42°C 1 min, 25 cycles</td>
<td>308 Czeczulin et al., 1999</td>
</tr>
</tbody>
</table>

None of the 55 E. coli isolates agglutinated with polyvalent O antisera, although 24 (43.6%) of the isolates showed the presence of the eaeA gene encoding intimin (Table II). Only 7 of these 24 eaeA-positive strains (29.2%) adhered to the epithelial cells in LA pattern. The remaining 17 eaeA-positive strains (70.8%) showed undefined (UD) pattern of adherence i.e. diffuse/localized (DA/LA) or localized/aggregative (LA/AA). FAS assay was positive for 19 out of the 24 E. coli strains (79.2%) and the reference strain (Fig. 1). The remaining 5 eaeA-positive but FAS-negative E. coli (20.8%) showed hemolytic activity on blood agar after 3 h of incubation and induced changes in the epithelial cells morphology (Fig. 1). All these 5 hemolytic isolates showed the presence of hlyA gene (Table II). E. coli α-hemolysin is a pore forming cytolsin targeting to the plasma membrane of red blood cells and a wide range of nucleated host cells, including intestinal epithelial cells. Damage to the cell membranes and the flow of ions triggers the disruption of cellular actin cytoskeleton, thus preventing the accumulation of polymerized F-actin beneath adhering bacteria (Menestrina et al., 2001). Based on the presence of the bfpA gene, 18 out of the 24 eaeA-positive E. coli (75%) were categorized as tEPEC (typical EPEC). The remaining 6 eaeA-positive, but bfpA-negative strains (25%) were considered as aEPEC (atyypical EPEC). Since most 18 eaeA-positive strains did not show adherence patterns of EPEC pathotype, therefore the genes encoded adhesins characteristic to diffusely adhering E. coli (DAEC) and enteroaggregative E. coli (EAEC) were evaluated. From a number of different Afa/Dr adhesins associated with DAEC pathotype the afaD gene of the afa operon encoding the AfaE-1 afimbrial adhesin and the daaA gene of the daa operon encoding F1845 fimbrin from a subfamily of Afa/Dr adhesins were selected. As EAEC pathotype characteristic genes, the aggR and aggB genes were chosen. The afaD and daaA genes were associated with 10 (41.7%) and 3 (12.5%) of the 24 eaeA-positive E. coli, respectively. Less commonly, the examined EPEC strains carried EAEC pathotype characteristic genes i.e. aggB and aggR that were present...
in 6 (25%) and 8 (33.3%) of isolates, respectively. Moreover, 6 of the 24 strains (25%) carried both genes specific for DAEC and EAEC pathotype. In contrast, none of the 7 of tEPEC strains (29.2%) that demonstrated LA pattern carried \(aggB\) and \(aggR\) genes.

In the study both, tEPEC and aEPEC strains were isolated from biopsy specimens from inflamed intestinal mucosa of children with chronic intestinal disorders accompanied by persistent diarrhea. The atypical strains constituted the minority of these strains. The association of tEPEC and aEPEC strains with intestinal mucosa may indicate their relationship with the inflammation and clinical symptoms or imply secondary colonization of the diseased mucosal tissue. In each of these cases, direct contact of tEPEC or aEPEC with intestinal epithelial cells can cause induction of characteristic histopathological AE lesions in the intestinal mucosa leading to the development of diarrhea. The ability of isolated EPEC to induce AE lesions was confirmed by positive FAS test for all but five hemolytic strains. All isolated EPEC strains were nontypeable with antisera specific for classical EPEC and majority of them carried genes characteristic to other than EPEC pathotypes, indicating that these strains differ somehow from EPEC and in that respect seem to be more similar to aEPEC. The presence of the \(afaD\) gene characteristic to DAEC pathotype that encodes both, adhesin and invasin AfaD (Jouve et al., 1997) among more than forty percent of EPEC, suggests that the acquired virulence factor can contribute to the internalization of these strains. Indeed, our preliminary study shows that many of these strains were internalized by the intestinal epithelial cells (data not shown). Moreover, nearly one third of the EPEC strains showed the presence of the \(aggR\) and \(aggB\) genes that are characteristic to EAEC pathotype. Yatsujangi et al. (2002) have also described EPEC strains isolated from diarrheal patients that represented typical tEPEC serotypes i.e. O126:NM and

---

**Table II**

Phenotypic and genotypic characteristics of twenty-four eaeA-positive strains of *E. coli*.

| *E. coli* strain | Adherence pattern | \(eaeA\) | \(bfpA\) | FAS | \(hlyA\) | hem\(^1\) | \(afaD\) | \(daaA\) | \(aggB\) | \(aggR\) | \(\# of strains (%)\) |
|------------------|------------------|--------|--------|-----|-------|--------|--------|-------|--------|-------|
| Typical EPEC     |                  |        |        |     |       |        |        |       |        |       |                  |
| EC35/1           | UD               | +      | +      | +   | -     | +      | +      | -     | +      | +     | +                 |
| EC37/1           | UD               | +      | +      | +   | -     | -      | +      | -     | -      | -     | +                 |
| EC41/5           | LA               | +      | +      | +   | -     | -      | -      | -     | -      | -     | -                 |
| EC42/1           | LA               | +      | +      | +   | -     | -      | -      | -     | -      | -     | -                 |
| EC44/1           | CDT              | +      | +      | ND  | +     | \(\beta\) | -      | -     | -      | -     | -                 |
| EC45/3           | UD               | +      | +      | +   | -     | -      | +      | +     | -      | -     | +                 |
| EC49/2           | LA               | +      | +      | +   | -     | -      | -      | -     | -      | -     | -                 |
| EC28/1           | UD               | +      | +      | +   | -     | -      | -      | +     | +      | -     | -                 |
| EC30/1           | UD               | +      | +      | +   | -     | -      | +      | -     | -      | -     | +                 |
| EC38/3           | UD               | +      | +      | +   | -     | +      | +      | +     | +      | +     | -                 |
| EC47/1           | LA               | +      | +      | +   | -     | -      | -      | -     | -      | -     | -                 |
| EC74P/4          | LA               | +      | +      | +   | -     | -      | -      | -     | -      | -     | -                 |
| EC25/1           | UD               | +      | +      | +   | -     | -      | -      | +     | +      | -     | +                 |
| EC36/1           | LA               | +      | +      | +   | -     | -      | -      | -     | -      | -     | -                 |
| EC84/1           | CDT              | +      | +      | ND  | +     | \(\beta\) | -      | +     | -      | -     | -                 |
| EC68P/5          | CDT              | +      | +      | ND  | +     | \(\beta\) | -      | -     | +      | +     | -                 |
| EC87/1           | UD               | +      | +      | +   | -     | -      | +      | +     | +      | -     | +                 |
| EC87P/5          | LA               | +      | +      | +   | -     | -      | -      | -     | -      | -     | -                 |
| Atypical EPEC    |                  |        |        |     |       |        |        |       |        |       |                  |
| EC86/1           | CDT              | +      | -      | ND  | +     | \(\beta\) | -      | -     | -      | -     | -                 |
| EC50/1           | CDT              | +      | -      | ND  | +     | \(\beta\) | -      | -     | -      | -     | -                 |
| EC53P/5          | DA               | +      | -      | +   | -     | -      | +      | +     | +      | +     | -                 |
| EC48/2           | UD               | +      | -      | +   | -     | -      | +      | -     | -      | -     | -                 |
| EC43/3           | LAL              | +      | -      | +   | -     | -      | -      | -     | -      | -     | -                 |
| EC46/1           | UD               | +      | -      | +   | -     | -      | +      | -     | -      | -     | -                 |

\(^1\) hemolytic activity was evaluated after 3 h and 24 h of incubation at 37°C; \(\beta\), clear zone of hemolysis visible after 3 h of incubation; LA, localized adherence; LAL, localized-like adherence; DA, diffuse adherence; UD, undefined mixed adherence e.g. localized/diffuse or localized/aggregative; CDT, cell-detaching strain; ND, not determined
pathotypes can contribute to chronic intestinal infections in children. The results of the study also raised the problem of the proper diagnosis of infections caused by nontypeable EPEC strains based only on the serologic determination of EPEC O serogroups, without detection of virulence factors these pathogens.

Acknowledgments
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Fig. 1. a) FAS- and eae-positive E. coli O26: H11 strain. Fluorescence accumulation (AE, attaching and effacing lesion) visible beneath adhering bacteria; b) FAS-positive E. coli isolated from child with Crohn’s disease. The magnified fragment of picture indicates AE lesion caused by the eae gene, but not eae or aggR gene associated with classical EPEC strains.

In conclusion, the results of the study indicated that nontypeable TEPEC and aEPEC strains carrying virulence factors characteristic to DAEC and EAEC

O111:NM and possessed the aggR gene, but not eae or bfpA genes associated with classical EPEC strains.

In conclusion, the results of the study indicated that nontypeable TEPEC and aEPEC strains carrying virulence factors characteristic to DAEC and EAEC...
In 1944, Christie and Atkins observed that arrow-shaped haemolysis (called “candle flame-shaped” or “arrowhead”) occurred when *Streptococcus agalactiae* (group B streptococcus, GBS) was grown in a zone of *Staphylococcus aureus* β-haemolysin activity (Christie et al., 1944; Darling 1975). This phenomenon was later called ‘CAMP test’, after the names of authors who first studied it (Christie, Atkins, and Munch-Petersen) and is currently used in microbiology laboratories as one among the most reliable methods to identify GBS, *Listeria monocytogenes* and *Rhodococcus equi* (Darling 1975; Munch-Petersen et al., 1945; Ramsey et al., 2010; Savini et al., 2013).

To perform the assay, reference *S. aureus* strains that produce β-haemolysin are streaked perpendicularly to the tested isolate, and arrowheads are observed after 24 h incubation. Indeed, any β-haemolysin-producing *S. aureus* isolate may be used (Darling 1975); among coagulase-positive microorganisms other than *S. aureus*, nevertheless, β-haemolysin is constitutively produced by *Staphylococcus (pseud)intermedius* (Ramsey et al., 2010; Devriese et al., 2005), while production by *Staphylococcus delphini* (that forms, together with *S. (pseud)intermedius*, the ‘*Staphylococcus intermedius* Group’) is labeled as undetermined (Devriese et al., 2005; Savini et al., 2013; Van Hoovels et al., 2006; Varaldo et al., 1988).

Therefore, we carried out a CAMP test by streaking *S. delphini* strain ATCC 49172 on a sheep blood plate perpendicularly to *S. agalactiae* (identification confirmed through latex agglutination) (Ramsey et al., 2010) and observed arrowhead (Fig. 1), surprisingly, meaning *S. delphini* β-haemolysin production. As a confirmation, strain ATCC 49172 was cultivated on horse and rabbit blood media, where the dark, *S. (pseud)intermedius*-like, α-haemolytic band (which is β-haemolysin-related) was not formed; it was instead clearly visible on sheep blood (Darling 1975; Dinges et al., 2000; Savini et al., 2013).

β-haemolysin, in fact, has been known to be highly haemolytic for sheep but not rabbit and horse erythrocytes, and is neither lethal in mice nor dermonecrotic in guinea pigs (Darling 1975; Dinges et al., 2000). It is secreted into the culture medium as an exotoxin by certain *S. aureus* strains, particularly those from animal habitats, as well as from all *S. (pseud)intermedius* isolates (Dinges et al., 2000; Savini et al., 2013). Although its role in disease pathogenesis is not completely

**CAMP test Detected *Staphylococcus delphini* ATCC 49172 β-Haemolysin Production**

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**Abstract**

Through a CAMP test, we first observed a *Staphylococcus delphini* strain (ATCC 49172) to release β-haemolysin. Production of the latter in this coagulase-positive species of the ‘*Staphylococcus intermedius* Group’, in fact, has been labeled to be undetermined, thus far. Of course, a wider number of strains have to be investigated in order to define whether this property is constitutive (like in *Staphylococcus (pseud)intermedius*), or strain-dependent (like in *Staphylococcus aureus*), and which clinical impact it has; nevertheless, we can state that *S. delphini* ATCC 49172 indeed produces this toxin.

**Key words:** *Staphylococcus delphini*, β-haemolysin, CAMP test
understood, thus far, high level expression in veterinary strains seem to indicate that producing organisms garner selective advantages from this toxin secretion (Dinges et al., 2000).

We first showed that an S. delphini strain produces β-haemolysin; however, this species is rarely isolated and further studies on a wider number of strains are needed, as soon as they are collected and identified, to define whether production is constitutive (like in S. (pseud)-intermedius) or strain-dependent (like in S. aureus). Nevertheless, we suggest, for the moment, that S. delphini β-haemolysin production be no more considered to be undetermined, but potential, and observed.

Acknowledgments
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