**INTRODUCTION**

*Acinetobacter baumannii*, a gram-negative nonfermentative coccobacillus, has in recent years emerged as one of the most troublesome pathogens for healthcare institutions worldwide. Its clinical significance has been propelled by its remarkable ability to upregulate or acquire resistance determinants, making it one of the alarm pathogens of the present time. These microorganisms have been implicated in a diverse range of infections and are a particular problem in intensive care and burn therapy units (Güdücüoğlu et al., 2005; Simor et al., 2002; van den Broek et al., 2006; Gordon and Wareham, 2010). Potential risk factors associated with the development of colonization or infection of hospitalized patients with *A. baumannii* strains include: prolonged length of hospital stay, hospital size (over 500 beds), underlying disease severity, invasive procedures and treatment (mechanical ventilation, urinary catheterisation, parenteral nutrition); exposure to broad-spectrum antimicrobial agents, such as carbapenems or third generation of cephalosporins, primary and acquired immunodeficiencies and age (Fournier and Richet, 2006; Playford et al., 2007; Wroblewska et al., 2007). *A. baumannii* has been implicated in a wide range of infections. The most common are: bacteremias, pneumonias, meningitis, urinary tract and wound infections (Maragakis and Perl, 2008). This coccobacillus
is intrinsically resistant to many antimicrobial agents, and moreover it is capable of developing resistance to most of them. General mechanisms of antimicrobial resistance include: low permeability of the outer-membrane, target-site modifications (e.g. PBP alternations), drug-inactivating enzymes (e.g. $\beta$-lactamases) and efflux-pumps (Poirier and Nordman, 2006; Zavascki et al., 2010). The rapid emergence and global dissemination of A. baumannii as a major nosocomial pathogen is remarkable and demonstrates its successful adaptation to the 21st century hospital environment. Invariably, one of the most alarming characteristics of this gram-negative pathogen is its ability to develop resistance to all available antibiotics including carbapenems which are drugs of choice in the treatment of severe infections (Garnacho-Montero and Amaya-Villar, 2010). Carbapenem resistance among A. baumannii strains can be mediated by two groups of $\beta$-lactamases such as: carbapenem-hydrolysing oxacillinases as well as molecular class B metallo-$\beta$-lactamases (Rossolini et al., 2007). However, the most widespread $\beta$-lactamases are carbapenem-hydrolysing oxacillinases belonging to molecular class D (CHDLs).

The OXA carbapenemases of Acinetobacter spp. are divided into four phylogenetic subgroups: OXA-23-like; OXA-40-like; OXA-51-like and OXA-58-like (Woodford et al., 2006). Chromosomally encoded enzymes belonging to OXA-51-like group are intrinsic to A. baumannii. Although it is clear that blaOXA-51-like genes are present in all of the isolates of A. baumannii and their detection could provide simple and convenient method of identification of the organism to the species level (Turton et al., 2006a; Stoeva et al., 2009). Acquired OXA carbapenemases (OXA-23-like, OXA-40-like and OXA-58-like) are both chromosomally and plasmid located enzymes. It has been documented that higher carbapenem hydrolysis rates may occur due to the acquisition of the ISAba1 elements upstream of the naturally occurring OXA-type carbapenemase (blaOXA-51-like) as well as acquired (blaOXA-23, blaOXA-58) encoding genes (Segal et al., 2005; Turton et al., 2006b; Perez et al., 2007; Peleg et al., 2008).

The aims of our study were to investigate:
1) the distribution of four subgroups of OXA carbapenemases;
2) occurrence of insertion sequence (ISAba1), in carbapenem-resistant Acinetobacter baumannii strains isolated from Intensive Care and Burn Therapy Units from hospital in Southern Poland.

MATERIALS AND METHODS

Bacterial strains
A collection of 104 non-repetitive (one per patient) carbapenem-resistant Acinetobacter baumannii isolates were investigated between 2005 and 2010, from 72 and 32 patients hospitalized respectively in ICU (21 females, 51 males) and BTU (7 females, 25 males) in Rydygier’s Hospital in Krakow, Poland. This 700-bed hospital contains 15 highly specialized wards. Most of the isolates were cultured between 2009 and 2010, 62 and 27 isolates, respectively. Samples from ICU included tracheal aspirates (74%), urine (13%) and other specimens (13%) while from BTU wounds (53%), blood (29%) and other specimens (18%).

Bacterial identification and antimicrobial susceptibility testing
Bacterial identification was performed by Vitek 2 Compact system (bioMerieux, France) with the GN cards, used according to the manufacturer’s instructions. Susceptibility of the isolates to antimicrobial agents was tested with cards AST-N022 and AST-N091 in Vitek 2 Compact system (bioMerieux, France). MICs (minimal inhibitory concentration) values were determined to: ticarcillin, ticarcillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cefazidime, cefepime, aztreonam, imipenem, meropenem, gentamicin, tobramycin, minocyclin, amikacin, ciprofloxacin, pefloxacin, ctrimoxazole and colistin. Results of the susceptibility testing were interpreted according to CLSI guidelines (2008). P. aeruginosa ATCC 27853 and E. coli ATCC 25922 were used as the reference strains. The antimicrobial susceptibility profiles of A. baumannii isolates were listed in Table 1. All strains selected for the study were carbapenem-resistant. The most active compound against these isolates was colistin (100% of susceptible strains). A total of 104 strains were determined as multidrug-resistant.
DNA isolation
Genomic DNA was isolated with the Genomic Mini (A&A Biotechnology, Poland). DNA quantification was performed by spectrophotometry at 260 nm. The purity of DNA was evaluated by the ratio of the absorbance at 260 and 280 nm (A260/A280) (Biometra, Germany).

Ampification of the bla_{OXA} genes by multiplex PCR method
All isolates were subjected to the multiplex PCR to detect bla_{OXA-51-like}, bla_{OXA-23-like}, bla_{OXA-40-like} and bla_{OXA-58-like} genes (described previously by Woodford et al., 2006). All primers used in this study were listed in Table 2. The PCR was carried out in thermocycler T personal (Biometra, Germany).

Screening for the presence of IS_{Aba1}
A. baumannii strains were assayed for IS_{Aba1} sequence by PCR with primers IS_{Aba1F} and IS_{Aba1R} (Table 2) giving rise to a 549 bp fragment.

TABLE 1 - Antimicrobial susceptibility of A. baumannii isolates.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>No. of resistant strains from ICU* (%)</th>
<th>No. of resistant strains from BTU# (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperacillin</td>
<td>72 (100%)</td>
<td>32 (100%)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>69 (96%)</td>
<td>29 (91%)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>70 (97%)</td>
<td>30 (94%)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>72 (100%)</td>
<td>32 (100%)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>72 (100%)</td>
<td>32 (100%)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>60 (83%)</td>
<td>32 (100%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>37 (51%)</td>
<td>8 (25%)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>72 (100%)</td>
<td>32 (100%)</td>
</tr>
<tr>
<td>Colistin</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*Intensive Care Unit; #Burn Therapy Unit

TABLE 2 - Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’ to 3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA51LF</td>
<td>TAA TGC TTT GAT CGG CCT TG</td>
<td>353</td>
<td></td>
</tr>
<tr>
<td>OXA51LR</td>
<td>TGG ATT GCA CTT CAT CTT GG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA23LF</td>
<td>GAT CGG ATT GGA GAA CCA GA</td>
<td>501</td>
<td></td>
</tr>
<tr>
<td>OXA23LR</td>
<td>ATT TCT GAC CGC ATT TCC AT</td>
<td></td>
<td>Woodford et al. (2006)</td>
</tr>
<tr>
<td>OXA24LF</td>
<td>GGT TAG TTG GCC CCC TTA AA</td>
<td>246</td>
<td></td>
</tr>
<tr>
<td>OXA24LR</td>
<td>AGT TGA GCG AAA AGG GGA TT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA58LF</td>
<td>AAG TAT TGG GGC TTG TGC TG</td>
<td>599</td>
<td></td>
</tr>
<tr>
<td>OXA58LR</td>
<td>CCC CTC TGC GCT CTA CAT AC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IS_{Aba1F}</td>
<td>CAC GAA TGC AGA AGT TG</td>
<td>549</td>
<td>Segal et al. (2005)</td>
</tr>
<tr>
<td>IS_{Aba1R}</td>
<td>CGA CGA ATA CTA TGA CAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
USA) in a final volume of 25 µl. The amplification conditions were following: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 45 s, 56°C for 45 s, 72°C for 3 min and final elongation at 72°C for 5 min. (Segal et al., 2005).

### PCR products detection

Amplicons were visualized under UV fluorescence (Vilber Lourmat, France) and following electrophoresis at 2% agarose gel prepared in TBE buffer and stained with ethidium bromide. Gels were photographed with a G5 digital camera (Canon, Japan). The size of PCR products was compared with molecular weight standard O’Gene Ruler 50 bp DNA Ladder Plus (Fermentas Life Sciences, Canada).

### RESULTS

Our study concerned a collection of 104 non-repetitive (one per patient) carbapenem-resistant Acinetobacter baumannii isolates recovered between 2005 and 2010 from 72 and 32 patients hospitalized respectively in ICU and BTU in Southern Poland. Detection of the four groups of OXA carbapenemases including intrinsic and acquired enzymes (bla\textsubscript{OXA-51-like}, bla\textsubscript{OXA-23-like}, bla\textsubscript{OXA-40-like} and bla\textsubscript{OXA-58-like}) by multiplex PCR and ISA\textsubscript{ba1} among example isolates of A. baumannii from ICU and BTU: 1, 50 bp molecular size marker (Fermentas Life Sciences, Canada); 2, A. baumannii ATCC 19606; clinical strains of A. baumannii from ICU: 3, p1000/9 (bla\textsubscript{OXA-51-like} positive); 4, p3827/9 (bla\textsubscript{OXA-51-like} and bla\textsubscript{OXA-23-like} positive); 5, p3749/9 (bla\textsubscript{OXA-51-like} and bla\textsubscript{OXA-40-like} positive); 6, p2252/9 (bla\textsubscript{OXA-51-like}, bla\textsubscript{OXA-23-like} and bla\textsubscript{OXA-40-like} positive); 7, p4077/9 (ISA\textsubscript{ba1} positive); 8, 50 bp molecular size marker; clinical strains of A. baumannii from BTU: 9, 252/6 (bla\textsubscript{OXA-51-like} positive); 10, p3750/8 (bla\textsubscript{OXA-51-like} and bla\textsubscript{OXA-23-like} positive); 11, p3445/9 (bla\textsubscript{OXA-51-like} and bla\textsubscript{OXA-40-like} positive); 12, p184/9 (ISA\textsubscript{ba1} positive); 13, negative control; 14, 50 bp molecular size marker.

#### TABLE 3 - Presence of genes encoding OXA enzymes and insertion sequence in A. baumannii strains isolated from ICU and BTU.

<table>
<thead>
<tr>
<th>ICU(^a)</th>
<th>BTU(^b)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>N(^a)</td>
<td>%</td>
<td>N(^a)</td>
</tr>
<tr>
<td>3</td>
<td>4.17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>25</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>63.89</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6.94</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Intensive Care Unit; \(^b\)Burn Therapy Unit; \(^*\)number of strains
51-like) and acquired OXA carbapenemases (OXA-23-like, OXA-40-like), whereas 5 (6.94%) strains had only blaOXA-51-like gene. Taking into account isolates cultured from patients hospitalized at BTU, we observed the presence of blaOXA-51-like and blaOXA-23-like genes in 28 (87.50%) while blaOXA-51-like and blaOXA-40-like genes in 2 (6.25%) strains. We also detected 2 (6.25%) isolates positive only for blaOXA-51-like gene.

Comparative analysis of occurrence of OXA encoding genes among ICU and BTU isolates revealed the difference between the analyzed wards. In our study we observed that the majority of strains cultured from ICU patients were positive for blaOXA-51-like and blaOXA-40-like genes (63.89%), while the most of isolates obtained from BTU possessed blaOXA-51-like and blaOXA-23-like genes (87.50%).

Summing up, all isolates included in this study were positive for blaOXA-51-like gene and negative for carbapenemases belonging to OXA-58 family. Among a total of 104 strains tested, 46 (44.23%) were blaOXA-51-like and blaOXA-23-like positive, while 48 (46.15%) were blaOXA-51-like and blaOXA-40-like positive. Three isolates (2.88%) contained simultaneously blaOXA-51-like, blaOXA-23-like, and blaOXA-40-like genes. In addition seven strains (6.73%) had only a blaOXA-51-like carbapenemase gene which gave a band of 353 bp. ISAba1 was found in all analyzed strains of A. baumannii from the hospital (Figure 1).

**DISCUSSION**

*Acinetobacter baumannii* is an important opportunistic bacterial pathogen responsible for serious infections in immunocompromised patients particularly in ICU and BTU. Surveillance of nosocomial *A. baumannii* infections has revealed trends of increasing antimicrobial resistance including carbapenem and multidrug resistance. The wide array of *A. baumannii* antimicrobial resistance mechanisms is represented by low permeability of the outer-membrane, target-site modifications, drug-inactivating enzymes and over-expression of multidrug efflux-pumps (Poirel et al., 2006a; Zavascki et al., 2010). Molecular class D OXA enzymes production is the major mechanism of carbapenem resistance among *A. baumannii* strains.

Naturally occurring OXA carbapenemases are OXA-51-like enzymes. Until today, up to 45 variants of OXA-51 (e.g. OXA-64-66, OXA-68-71, OXA-78-80, OXA-82, OXA-86, OXA-92, OXA-104-112) have been identified in *A. baumannii* isolates from medical centers worldwide (Turton et al., 2006a; Walsh 2010; Zavascki et al., 2010). Multiplex PCR performed in our study detected 104 carbapenem-resistant *A. baumannii* clinical isolates positive for blaOXA-51-like gene. A survey by Wroblewska also demonstrated possession of blaOXA-51-like gene among 110 strains of *A. baumannii* (Wroblewska et al., 2007). Similar results were also obtained by Heritier et al. (2005b), Turton et al. (2006a), Woodford et al. (2006), Evans et al. (2008) and Taherikalani et al. (2009). Our study confirmed that detection of blaOXA-51-like can be used as simple and reliable way to identify *A. baumannii* (Turton et al., 2006b; Evans et al., 2008; Stoeva et al., 2009).

Insertion sequence ISAba1, which has 11-bp inverted repeat sequences (IRs) flanked by 9-bp direct repeats of the target sequence, has been identified in *A. baumannii* and as one of many IS elements contains promoters that play a role in the expression of antibiotic resistance genes (Segal et al. 2005).

In our study all 104 carbapenem-resistant isolates were PCR positive for ISAba1 (Turton et al., 2006b; Stoeva et al., 2008). Turton and other authors have proposed that insertion of ISAba1 upstream of the blaOXA-51-like genes may provide the promoter to enhance gene expression potentially contributing to increased levels of resistance to carbapenems (Turton et al., 2006b; Evans et al., 2008; Zavascki et al., 2010).

We conclude that the presence of ISAba1 upstream of the blaOXA genes among strains isolated from hospital in Krakow deserves further investigation.

Performing PCR mapping described by Turton et al. (2006b) would clarify the role of the ISAba1 in carbapenem resistance among strains tested in this study. Bratu et al. (2008) also observed the association of the promoter sequence ISAba1 with the blaOXA-51-like carbapenemase among carbapenem-resistant strains of *A. baumannii*, however this association was also present in several of tested isolates susceptible to imipenem. These findings suggest the need of supplementing our
group of strains with non-carbapenem-resistant isolates in further studies.

Acquired carbapenem-hydrolyzing class D β-lactamases can be divided into three clusters, based upon the variant sequence homology: OXA-23 (including OXA-23, OXA-27, OXA-49 and OXA-73); OXA-40 (including OXA-25, OXA-26, OXA-40/24 and OXA-72) and OXA-58 (including OXA-58, OXA-96 and OXA-97) (Poirel et al., 2010).

Detection of OXA-23 enzyme in Acinetobacterbaumannii strain isolated in Scotland was the first report of acquired class D β-lactamase with carbapenemase activity (Scaife et al., 1995; Poirel et al., 2010). Enzymes belonging to OXA-23 subgroup are spread to many locations worldwide including: Europe (Mugnier et al., 2010; Grosso et al., 2011; Towner et al., 2011), Asia (Mendes et al., 2009a; Taherikalani et al., 2009) and Southern America (Carvalho et al., 2009); and they have been identified as either chromosomal - or plasmid-mediated. Other OXA-23 enzymes such as: OXA-49, OXA-73 were identified in Acinetobacterbaumannii and Klebsiella pneumoniae, respectively, whereas OXA-133, OXA-134 were identified in Acinetobacter radioresistance and Acinetobacter radioreistance (Poirel et al., 2008; Mendes et al., 2009b; Poirel et al., 2010).

A report from Polish hospital did not reveal OXA-23-like enzymes among analyzed A. baumannii strains (Wroblewska et al., 2007). In our study 46 (44.23%) strains were blaOXA-23-like positive.

The OXA 40 group has been reported in Portugal, Spain (Quinteira et al., 2007; Ruiz et al., 2007), Iran (Taherikalani et al., 2009) and the United States (Lolans et al., 2006; Qi et al., 2008). While OXA-25 and OXA-26 appear to be the dominant acquired CHDLs in Europe, OXA 40/24 are identified both in Europe and United States (Zavascki et al., 2010). Contrary to results obtained by Wroblewska et al. (2007) we reported the presence of blaOXA-40-like gene among 48 (46.15%) of analyzed strains.

The first description of OXA-58-like enzymes has been reported in multidrug-resistant A. baumannii strain isolated in France (Poirel et al., 2005).

In addition strains producing OXA-58 derivatives were found in isolates recovered from Italy, Belgium, France, Greece, Iran, the United States and Argentina (Héritier et al., 2005a; Poirel et al., 2005; Marqué S. et al., 2005; Bertini et al., 2006; Bogaerts et al., 2006; Coelho et al., 2006; Castanheira et al., 2008; Poirel et al., 2010; Taherikalani et al., 2009).

We did not reveal any OXA-58-like encoding genes among isolates from Krakow Hospital, similar results were obtained for 110 isolates from Warsaw hospital (Wroblewska et al., 2007). Since 2000 there has been an increase in MIC values for imipenem for A. baumannii isolates from ICU patients, ranging from 38.9% to 43.8% of resistant strains (Fleischer et al., 2002; Wroblewska et al., 2006).

Since Acinetobacter baumannii is increasingly important opportunistic pathogen that affects patients hospitalized particularly in intensive care and burn units, we performed comparative analysis of occurrence of OXA encoding genes among ICU and BTU isolates. Our studies revealed the difference between the analyzed wards, the majority of strains cultured from ICU patients were positive for blaOXA-51-like and blaOXA-40-like genes (63.89%), while the most of isolates obtained from BTU possessed blaOXA-51-like and blaOXA-25-like genes (87.50%). There is limited data in literature concerning such approach and this is the first study of OXA carbapenemases in A. baumannii from hospital in Southern Poland.

Our report revealed that the presence of OXA enzymes and insertion sequence (ISAba1), among 104 imipenem- and meropenem-resistant clinical isolates of A. baumannii isolated from ICU and BTU might be responsible for carbapenem resistance.

Polish data concerning carbapenem resistance among A. baumannii strains are limited, while reduced susceptibility to these agents is a matter of increasing clinical concern worldwide. Our results highlight the need to monitor resistance levels and mechanisms among A. baumannii strains.

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REFERENCES


EMERGING CARBAPENEMASES: A GLOBAL PERSPECTIVE


