The Study of Prevalence and Expression of Efflux Pump Genes in Acinetobacter baumannii Strains Resistant to Aminoglycosides in Sari (Iran.)

Mohtaram Nasrolahei1,2, Bahador Zahedi1, Neda Jalalvand2, Hossein Saghi3 and Davoud Esmaeili3*

1Department of Microbiology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.
2Department of Genetic, Science and Research Branch, Islamic Azad University, Tabriz, East Azerbaijan, Iran
3Applied Microbiology Research center, and Microbiology Department, Baqiyatallah University Medical of Sciences, Iran.

(Received: 18 May 2014; accepted: 02 August 2014)

Multidrug resistant strains of Acinetobacter baumannii (MDR-AB) have emerged as alarming nosocomial pathogens among patients admitted to Intensive Care Unit and burned patients. The aim of this study was to determine the susceptibility of A. baumannii isolates, the carbapenems resistance patterns bla \textsubscript{OXA-23} and IS\textsubscript{Aba} elements of A. baumannii isolates among burned patients in Sari, Iran. During the course of this investigation, our aim was to examine prevalence and Expression of ade A and ade R Efflux Pump genes in Acinetobacter baumannii Strains resistant to Aminoglycosides by PCR and RT-PCR methods. Multidrug resistant strains of Acinetobacter baumannii (MDR-AB) have emerged as an important cause of hospital acquired infection among patients admitted to Intensive Care Unit and burned patients. A total of 100 isolates were identified as Acinetobacter baumannii from patients that were admitted to Intensive Care Unit and burned patients in Sari, Iran. During 2013, the isolates were tested for the determination of resistance to Aminoglycosides by Disk Diffusion method according to the CLSI protocol. On the next step, we detected ade A and ade R Efflux Pumps gene in Acinetobacter baumannii that were resistant to Aminoglycosides by PCR method. Finally we investigated Expression of ade A and ade R Efflux Pump genes by RT-PCR method. Among the 62 (62%) strains that showed resistance to Aminoglycosides, in 52 (83%) strains, we detected Ade A and Ade R genes. After RT-PCR was found that only in 3 strains of Acinetobacter baumannii ade A gene, and in 6 strains ade R gene have not been expressed.

Key words: A. baumannii, Efflux Pumps, Aminoglycoside, RT-PCR, PCR.
A survey by the Health Protection Agency in England found that patients with Acinetobacter bacteremia were generally aged > 50 years, that the majority were male, and that 5% were hospitalized in general wards and 54% in ICUs. Risk-factors have been defined in many studies, and are essentially the same as those identified for other opportunistic bacteria. One study reported sepsis and or septic shock in 19% of patients with Acinetobacter bacteremia. This observation highlighted the true pathogenicity of a few strains, with a crude mortality rate of c. 42%. An attributable mortality rate of 7.8% found in one survey was related to a delay in the initiation of appropriate therapy. Aminoglycoside agents, such as tobramycin and amikacin, are therapeutic options for infection with multidrug-resistant Acinetobacter isolates that retain susceptibility. These agents are usually used in conjunction with another active antimicrobial agent. Many multidrug-resistant Acinetobacter isolates retain intermediate susceptibility to amikacin or tobramycin; resistance to this class of agents is increasingly associated with aminoglycoside-modifying enzymes or efflux pump mechanisms. Active efflux is now recognized as an important component of bacterial resistance to most of classes of antibiotics. This mechanism is mediated by efflux pumps, which are membrane-associated active transporters promoting the extrusion of toxic compounds, including antibiotics, from the cells. Bacterial drug efflux transporters are currently classified into five families: 1. the major facilitator superfamily (MFS) 2. The adenosine triphosphate (ATP)-binding cassette (ABC) superfamily 3. The small multidrug resistance (SMR) family 4. The resistance-nodulation-cell division (RND) superfamily and 5. the multidrug and toxic compound extrusion (MATE) family. In Acinetobacter baumannii AdeB is the multidrug transporter protein, AdeA is the MFP and AdeC is the OMP. The efflux transporter (AdeB) captures its substrates either from within the phospholipid bilayer of the inner membrane or the cytoplasm and then transport them into the extracellular medium via OMP (AdeC). The periplasmatic protein AdeA mediates in the cooperation between AdeB and AdeC components (Figure 1).

**MATERIALS AND METHODS**

**Bacterial Strain**

During a One year period, 100 non-duplicate A. baumannii were isolated from patients that were admitted to Intensive Care Unit and burned patients with proved nosocomial infections in Sari, (Iran). The strains were isolated from the trachea and burned skin. Patients had no bacterial infections at the time of Admitted to hospital. All the samples were confirmed as A. baumannii by biochemical Tests.

**Antimicrobial susceptibility tests**

Susceptibility to Aminoglycosides antimicrobial agents was determined by the disc-diffusion method on Mueller Hinton agar recommended by the guidelines of Clinical and Laboratory Standards Institute (CLSI). Briefly, 0.1 ml of a suspension of the test microorganism (1.5 × 10^8 cfu/ml) was spread on Mueller-Hinton Agar (diameter, 90 mm) (Merck), by the disc diffusion method for the following antimicrobial agents (Mast) with their concentration given in parentheses: Streptomycin (10 µg), Tobramycin (10 µg), Gentamicin (10 µg), Kanamycin (10 µg), were then placed on the agar plate and incubated at 37°C for 24h. The diameters of the zones of inhibition were measured and reported in mm. Then we have separated resistant strains for detected Ade A and Ade R genes by PCR and Evaluate the expression of them by RT-PCR

**DNA Extraction and PCR Assay**

DNA extraction was carried out by commercial DNA extraction kit (CinnaGen, Iran). The presence of Ade A and Ade R genes was detected by PCR. PCR was performed in a standard enzyme Taq DNA polymerase. Target genes and corresponding primers used For PCR amplification are listed in the following table:

<table>
<thead>
<tr>
<th>TM (°C)</th>
<th>Sequence 5’ to 3’</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>F: 50°C</td>
<td>F:</td>
<td>Ade A</td>
</tr>
<tr>
<td>R: 56°C</td>
<td>TGGTTGCCATCGTATTGGTA R: TCAGGCTCTAGCCGATGTC</td>
<td></td>
</tr>
<tr>
<td>F:48°C</td>
<td>F:</td>
<td>Ade R</td>
</tr>
<tr>
<td>R:48°C</td>
<td>TGGCGATTGCTATTTCAAATG R: TGCCGCCAATTCTTTATTC</td>
<td></td>
</tr>
</tbody>
</table>
For PCR reaction we used CinnaGen Master Mix and reactions were performed in a final volume of 25 µL According to following table:

<table>
<thead>
<tr>
<th>Primer F</th>
<th>1µl (10 pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer R</td>
<td>1 µl (10 pmol)</td>
</tr>
<tr>
<td>DNA Template</td>
<td>2 µl (50 ng)</td>
</tr>
<tr>
<td>Master Mix</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>DDW</td>
<td>8.5 µl</td>
</tr>
</tbody>
</table>

The PCR cycles for ade A and ade R genes were According to following table:

<table>
<thead>
<tr>
<th>No of complete cycles</th>
<th>Final extension</th>
<th>Extension</th>
<th>Annealing</th>
<th>Denaturation</th>
<th>Initial healing</th>
<th>PCR reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>30°C,32</td>
<td>72°C,102</td>
<td>72°C,12</td>
<td>55°C,302</td>
<td>95°C,30º</td>
<td>Ade A</td>
</tr>
<tr>
<td>40</td>
<td>30°C,32</td>
<td>72°C,102</td>
<td>72°C,12</td>
<td>48°C,302</td>
<td>95°C,30º</td>
<td>Ade R</td>
</tr>
</tbody>
</table>

The amplified products were analyzed by electrophoresis on 1% agarose gel (CinnaGen) containing 0.1 g of ethidium bromide per ml in TAE buffer. The PCR product was visualized under UV light and photographed. 1-kb DNA ladder or the 100-bp DNA ladder (CinnaGen, Iran) was used to assess PCR product size. DNA from a clinical isolate of *P. aeruginosa* was used as a negative control in the PCR reaction.

**RESULTS**

In total, 100 Acinetobacter baumannii were isolated from patients that were admitted to
Intensive Care Unit and burned patients in Sari, Iran. During 2013, we observed that, 62 (62%) isolates showed resistance to Aminoglycosides and maximum resistance against Streptomycin (90%), Gentamicin (83%), Tobramycin (83%) and kanamycin (80%). Among the 62 strains that showed resistance to Aminoglycosides, in 52 (83%) strains we detected ade A and ade R genes. After RT-PCR We Found that only in 3 strains of Acinetobacter baumannii ade A gene, and in 6 strains ade R gene have not been expressed.

The challenges of treating multidrug-resistant bacteria continue to be at the forefront of the clinician’s practice in caring for hospitalized patients. Acinetobacter baumannii has proven to be an increasingly important and demanding species in health care associated infections. The ability of A. baumannii to survive for extended periods on environmental surfaces are notorious and are likely important for transmission within the health care setting. Multidrug resistance is common with health care–associated A. baumannii infections.

DISCUSSION

As a primarily nosocomial pathogen, A. baumannii causes a wide-range of infections in immunocompromised people, most often pneumonia and bloodstream infections, and, in contrast with most other Acinetobacter spp., it is rarely isolated outside of the hospital environment. The treatment of A. baumannii infections has become increasingly difficult due to the widespread dissemination of multi- and pan-drug resistant strains.

Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment. These proteins are found in both Gram-positive and -negative bacteria as well as in eukaryotic organisms. Pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds (including antibiotics of multiple classes); such pumps can be associated with multiple drug resistance (MDR). In the prokaryotic kingdom there are five major families of efflux transporter: MF (major facilitator), MATE (multidrug and toxic efflux), RND (resistance-nodulation-division), SMR (small multidrug resistance) and ABC (ATP binding cassette).

AdeABC efflux pump, belonging to the resistance–nodulation-cell division family (RND) efflux pump, has a broad range of substrates, such as aminoglycosides, tetracycline, erythromycin, chloramphenicol, trimethoprim, fluorquinolones, and tigecycline and so on.

Our study showed that, Efflux pumps play an important role in drug resistance to the aminoglycoside in Acinetobacter baumannii resistant to treatment.

REFERENCES

3. Chul Hee Choi1, Jun Sik Lee1, Yoo Chul Lee1, Tae in Park, and Je Chul Lee. Acinetobacter baumannii invades epithelial cells and outermembrane protein A mediates interactions with epithelial cells. BMC microbiology. 2008; 8:216.
8. Piotr Wieczorek1, Pawe Sachal, Tomasz Hauschild, Marcin orawski, Magorzata Krawczyk, Tryniszewskai. Multidrug resistant Acinetobacter baumannii the role of AdeABC (RND family) efflux pump in resistance to antibiotics. folia histochemica et cytobiologica, 2008; 46(3) 257-267.
of Acinetobacter Infections. *Clinical infectious diseases*. 2010; 51(1):79–84

