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

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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 $^{OXA-23}$ and IS_{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.

The genus Acinetobacter is now defined as including gram-negative coccobacilli, with a DNA G+C conten of 39 to 47 mol%, that are strictly aerobic, nonmotile, catalase positive and oxidase negative[1]. Interest in Acinetobacter spp. has been growing for the past 30 years. One of the main reasons for the present increased interest in this genus is the emergence of multiresistant strains, some of which are pan-resistant to antibiotics that suddenly cause an outbreak of infection involving several patients in a clinical unit[2]. Genus Acinetobacter are important opportunistic pathogens in hospital-acquired infections. They cause various types of human infections, including pneumonia, wound infections, urinary tract infections, bacteremia, and meningitis. Of the currently known 31 Acinetobacter species, Acinetobacter baumanniiis the most prevalent in

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clinical specimens[3]. 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 D 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[4]. 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[5]. 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[6]. 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[7].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[8] (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. baumanni 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 (10µg), 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:

TM (C')	Sequence 5' to 3'	Primer
F: 50°C R: 56°C	F: TGGTTGCCATCGTATTGGTA R: TCAGGCTCTAGCCGATGTC	Ade A
F:48°C R:48°C	F: TGCGATTCGCTATTCAAATG R: TGCCGCCAAATTCTTTATTC	Ade R

For PCR reaction we used CinnaGen Master Mix and reactions were performed in a final volume of 25 µL According to following table:

Primer F	1µl (10 pmol)
Primer R	1 µl(10 pmol)
DNA Template	2 µl (50 ng)
Master Mix	12.5 µl
DDW	8.5 µl

The PCR cycles for ade A and ade R genes were According to following 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.After PCR, to perform RT-PCR, we separate the isolates which contain Ade A and Ade R genes.

No of complete cycles		Final extension	Extension	Annealing	Denaturation	Initial healing	PCR reaction
40	30°C,32	72ºC,102	72ºC,12	55ºc,302	95ºC,30º	95ºC,32	Ade A
40	30°C,32	72ºC,102	72ºC,12	48ºc,302	95ºC,30º	95ºC,32	Ade R

RNA Extraction and RT-PCR

RNA extraction was carried out by commercial RNA extraction kit (CinnaGen, Iran) according to the manufacturers' instructions.

For first strand cDNA synthesis we used commercial 2-steps RT-PCR kit (Vivantis, Malaysia).

In the first stage we denatured RNA samples (RNA + Primer + dNTP) According to following table:

1µl (10 pmol)	Primer R
1µl (10 pmol)	Primer F
1µl (10 µM)	dNTP
5µl	Nuclease free water
2µl	Sample (RNA)

Incubate 65°C for 5 min and chill on ice for 2 min. In the second stage we add 10µl cDNA synthesis Mix According to following table:

2µl (1X)	M-Mulv Buffer
0/1µl (1U)	M-MulV Reverse Transcriptase
8µl	Nuclease free water

Incubate at 42°C for 60 min. In the third stage Incubate at 85°C for 5min to stop the reaction and now we have cDNA the micro-tubes. Finally we used 2µl as template for PCR reaction According to following tables:

1µl (10 pmol)	Primer F
1 µl (10 pmol)	Primer R
2 µl (50 ng)	Sample
12.5 µl	Master Mix
8.5 µl	DDW

No of complete cycles		Final extension	Extension	Annealing	Denaturation	Initialhealing	PCR reaction
40	30°C,32	72ºC,102	72ºC,12	55ºc,302	95ºC,30º	95ºC,32	Ade A
40	30°C,32	72ºC,102	72ºC,12	48ºc,302	95ºC,30º	95ºC,32	Ade R

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[9].

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[10]

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)[11]

AdeABC efflux pump, belonging to the resistance–nodulation-cell division family (RND) efflux pump, has a broad range of substrates, such as aminoglicosides, tetracycline, erythromycin, chloramphenicol, trimethoprim, fluorquinolones, and tigecycline and so on[12]

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

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