Molecular Characterization and Antibiotic Resistance Pattern of Nosocomial Clinical Isolates in Southeast of Iran

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ABSTRACT

**Background and objectives:** Nosocomial infections caused by antibiotic resistant bacteria is a life threatening health challenge. This study aimed to determine the frequency of antibiotic resistance genes in clinical isolates from hospitals of Zahedan, southeast of Iran.

**Methods:** Overall, 818 isolates were collected from different hospital wards. The isolates were identified using conventional microbiological and biochemical tests. Antibiotic susceptibility pattern was assessed by agar disc diffusion method and determination of minimum inhibitory concentration of a number of antibiotics. Multiplex PCR was performed using specific primers for the detection of resistance genes.

**Results:** The most common species were *Staphylococcus aureus* (25%), *Klebsiella pneumoniae* (22%) and *Pseudomonas aeruginosa* (14%). The rate of methicillin resistance among *S. aureus*, *S. epidermidis* and *S. saprophyticus* was 60%, 43% and 24%, respectively. In addition, 28.5% of enterococci isolates were vancomycin resistant. Among gram-negative bacteria, 45% of *A. baumannii* and 24% of *P. aeruginosa* were identified as ESBL. A high level of resistance to ampicillin (96%), cefotaxime (89%), gentamicin (89%) and sulfamethoxazole-trimethoprim (60%) was observed in *K. pneumoniae*.

**Conclusion:** Our results highlight the urgent need for an eradication program and a surveillance plan for preventing increased emergence of antibiotic resistant bacteria in the study area.

**Keywords:** Bacterial Infections, Drug resistance, Zahedan.
INTRODUCTION
Nosocomial infection is an infection that is acquired in a hospital. Surgery, transplantation and hospitalization in the pediatric ward, intensive care unit (ICU) and cardiac care unit (CCU) are some of the risk factors for nosocomial infections (1, 2). A large number of both gram-positive and gram-negative bacteria including *Staphylococcus* spp., *Enterococcus* spp., *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacteriaceae* can cause various hospital-acquired infections such as urinary tract infection, bacteremia, wound infection, gastrointestinal infection, etc. (3-6). Furthermore, the emergence of methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), extended-spectrum β-lactamase (ESBL) *Enterobacteriaceae* spp. and multi drug resistant (MDR) strains has become a major challenge to treatment of nosocomial infections (7-9). Hence, the World Health Organization (WHO) has released a global priority list of antibiotic-resistant bacteria to help prioritize research and development of new and effective antibiotic treatments (8, 9). The primary objective of this study is to evaluate the prevalence of nosocomial infections and determine involved bacteria and their antibiotic resistance genes in Zahedan, southeast of Iran.

MATERIAL AND METHODS
Bacterial isolates associated with nosocomial infections were collected from four hospitals in Zahedan (southeast of Iran) during 2014-2016. The samples were collected from different wards including ICU, CCU, pediatric ward, internal medicine ward and general surgery and transplantation ward. This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the ethics committee of the Zahedan University of Medical Sciences. The bacterial isolates were identified by culture, gram staining, morphological assessment and biochemical tests such as catalase, oxidase, oxidation/fermentation, salt tolerance, coagulase reaction, hemolysis, esculin hydrolysis, growth on MacConkey agar, IMViC, decarboxylases, sugar fermentation, malonate, DNase, motility, indole production, hydrogen sulfide production and reaction in triple sugar iron agar, etc. All isolates were then stored in Mueller Hinton broth with 30% glycerol at -70 °C for analysis. All culture media and reagents were purchased from Merck Co., Germany. Antibiotic susceptibility was assessed by the disk diffusion method. Minimum inhibitory concentration (MIC) of the following antibiotics was determined using E-test strips (Liofilchem, Italy) according to the Clinical and Laboratory Standards Institute (CLSI) recommendations: tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, oxacillin, methicillin, piperacillin and piperacillin/tazobactam, azteronam, ceftriaxone, ceftazidine, cefepime, cefotaxime, cefexime, ciprofloxacin, gatifloxacin, norofloxacin, levofloxacin, ofloxacin, nalidixic acid, cotrimoxazole, imipenem, meropenem, gentamicin, amikacin, tobramycin, kanamycin, fosfomycin, vancomycin, linezolid, nitrofurantoin, quinupristin/dalfopristin and colistin. All antibiotic disks were purchased from Mast Co., UK.

The double disk synergy test was performed to evaluate ESBL production. In this test, augmentin (20 μg amoxicillin and 10 μg clavulanic acid) and third-generation cephalosporins (ceftazidime 30 μg, ceftriaxone 30 μg, cefotaxime 30 μg) disks are kept 30 mm apart, center to center, on inoculated Mueller-Hinton agar (MHA). A clear extension of the edge of the inhibition zone of cephalosporin towards augmentin disk is interpreted as positive for ESBL production. After incubation at 35±2 °C for 18h, ESBL positivity was confirmed by observing increased inhibition zone around one of the four antibiotic disks toward amoxicillin–clavulanic acid. Susceptibility cut-offs were considered according to the CLSI. *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *E. coli* ATCC25922, *P. aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC700603, *Enterococcus faecalis* ATCC29212, *S. aureus* ATCC3591 and *S. aureus* ATCC 25923 were used as control strains. After 18h at 35±2 °C, chromosomal and plasmid DNA were extracted using a mericon DNA bacteria kit (Qiagen, Germany) according to the manufacturer’s instructions. The quality and quantity of the extracted DNA were tested by gel electrophoresis and spectrophotometry, respectively. Genotypic identification of each clinical isolate was...

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carried out by PCR using specific primers (Table 1) and results were confirmed by biochemical tests. Then, multiplex PCR was used for detection of antibiotic resistance genes using specific primers (Table 2) for each species. Multiplex PCR was performed in a final volume of 25 µL containing 1 µL of each primer (0.3 mM), 12 µL of PCR Master Mix (Fermentase, Denmark), 0.05 U/µL Taq DNA polymerase, 0.4 mM dNTPs, 4 mM MgCl₂ and 1 µL template DNA. The cycling conditions for 10 minutes, 35 cycles of denaturation at 94 °C for 30-60 seconds, annealing at 56 °C for 35 seconds, extension at 72 °C for 30-60 seconds and final extension at 72 °C for 5-10 minutes.

DNA sequencing was performed by Macrogen Co., South Korea for appraising highest purity and accuracy of antibiotic resistance genes. The obtained sequences were aligned and compared to data available at GenBank (NCBI).

Figure 1 - Frequency of clinical isolates collected from hospitals of Zahedan, Iran. UC: urine culture; BC: blood culture; W: wound infection; Uca: urine catheters; Ett: endotracheal tube; csf: cerebrospinal fluid

Figure 2 - Antimicrobial susceptibility patterns among gram-positive isolates. VAN: vancomycin; AMP: ampicillin; IMI: imipenem; SYN: quinupristin/dalfopristin (Synercid®); ERY: erythromycin; TET: tetracycline; CHL: chloramphenicol; FOS: fosfomycin
Figure 3-Antimicrobial susceptibility patterns among gram-negative isolates

Table 1- Partial 16S rDNA sequences of the specific primers used in the study

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Primer sequence (5′–3′)</th>
<th>Product size (bp)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA</td>
<td>108 bp</td>
<td>1</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>ATC AAA AAG TTG GCG AAC CTT TTC A CAA AAG AGC GTG GAG AAA AGT ATC A</td>
<td>124 bp</td>
<td>1</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>AAC GGG CGT CTC GAT AGA AAA AAC GGG CGT CCA CAA AAT CA</td>
<td>380 bp</td>
<td>1</td>
</tr>
<tr>
<td><em>E. faecalis</em> ddT</td>
<td>5′-ATCAAGTACAGTTAGTCTTTATTAG-3′ 5′-ACGATTCAAGCTAATGGAATCAGT-3</td>
<td>941 bp</td>
<td>4</td>
</tr>
<tr>
<td><em>E. faecium</em> ddT</td>
<td>5′-TTGAGGCAGACGATCAGGCAG-3′ 5′-TATGACAGCGACTCCGATCAGTT-3</td>
<td>658 bp</td>
<td>4</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>GCAAGTGACGGCTAGCATACAGCAGTGTCATCTCTTCTC</td>
<td>260 bp</td>
<td>17</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>AATTTACAGTGGCAATAGGATCCGCAGGCTACGACGAGGATACAGG</td>
<td>722 bp</td>
<td>29</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>ATGATCGTACAAATTGCTCGG GTCATGAAACCAGCCAGTC</td>
<td>600 bp</td>
<td>29</td>
</tr>
</tbody>
</table>
RESULTS

Of 818 nosocomial isolates collected from different wards, 335 (41%) were staphylococci species consisting of 203 (25%) S. aureus, 77 (9%) S. epidermidis, 53 (7%) S. saprophyticus and two other staphylococci species. Other isolates detected in clinical samples were E. faecalis (n=65, 8%), E. faecium (n=26, 3%), K. pneumoniae (n=179, 22%), P. aeruginosa (n=112, 14%) and A. baumannii (n=101, 12%).

According to the results of the antimicrobial susceptibility testing, the frequency of methicillin resistance (mecA gene) among S. aureus, S. epidermidis and S. saprophyticus isolates was 60%, 43% and 24%, respectively. Nevertheless, no isolate was resistance to vancomycin (vanA and vanB), meropenem, chloramphenicol, ofloxacin and norofloxacin (Table 3). The highest and lowest resistance rates in both species were recorded against tetracycline and linezolid, respectively. We isolated 112 P. aeruginosa strains from samples collected from nosocomial infections. The highest and lowest resistance rates in these isolates were recorded against cefotaxime (50%) and collistin (1%), respectively. In addition, resistance rate against ceftriaxone, ceftazidime, cefepime, cefexime, ciprofloxacin, imipenem, meropenem, aztreonam, and gentamicin was 30%, 22%, 10%, 14%, 3%, 32%, 15%, 5% and 20%, respectively. According to results of the DDST, 24% of all P. aeruginosa isolates were ESBL strains.
Table 3- The frequency of multidrug resistance among clinical isolates from hospitals of Zahedan, Iran

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of isolates</th>
<th>Multi drug resistance pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td></td>
<td>11 OXA-ERY-TET, 4 OXA-GM-KAN-ERY, 3 OXA-GM-KAN-TET, 3 OXA-ERY-TET-CLI, 1 OXA-GM-ERY-TET-SXT, 1 OXA-GM-KAN-ERY-TET</td>
</tr>
<tr>
<td>E. faecium</td>
<td>1</td>
<td>1 VAN-ERY-TET-GM-IMI-AMP-LIN-NIT-PIP-SYN-CIP-CHL, 1 VAN-ERY-TET-GM-IMI-AMP-LIN-NIT-PIP-CIP</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>2</td>
<td>1 AMP-CHL-IMI-IMP, 1 AMP-CHL-IMI-IMP</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>2</td>
<td>1 AMP-CHL-IMI-IMP, 1 AMP-CHL-IMI-IMP</td>
</tr>
<tr>
<td>A. baumannii</td>
<td>2</td>
<td>1 AMP-CHL-IMI-IMP, 1 AMP-CHL-IMI-IMP</td>
</tr>
</tbody>
</table>

VAN: vancomycin; AMP: ampicillin; IMI: imipenem; ERY: erythromycin; TET: tetracycline; CHL: chloramphenicol; FOS: fosfomycin, LIN: linezolid; NIT: nitrofurantoin; PIP: piperacillin; AZT: azteronam; CTX: ceftriaxone; CAZ: ceftazidime; CEF: ceftaxime; CEF: cefuroxim, CIP: ciprofloxacin, norfloxacin, levofloxacin, ofloxacin, nalidixic acid; SXT: cotrimoxazole; MER: meropenem; GM: gentamicin; AN: aminoglycosides; TOB: tobramycin; KAN: kanamycin
DISCUSSION

Nosocomial infections are associated with significant risk of mortality and increased treatment cost (10). Microorganisms that are able to survive in the hospital environment can enter the body through wounds, catheters and ventilators. Gram-negative bacilli are important pathogens that are becoming increasingly resistant to most available antibiotics (10, 11).

In this study, 392 gram-negative and 424 gram-positive bacteria were isolated from samples collected from different parts of four hospitals in Zahedan, Iran. The most prevalent gram-negative and gram-positive bacteria were *K. pneumoniae* and *S. aureus*, respectively. One of the main causes of nosocomial infections is *A. baumannii*, which has recently become resistant to majority of β-lactam antibiotics (12, 13). The results of the present study showed that 24% of *Acinetobacter* strains isolated from outpatients in Zahedan produced ESBL. As seen in figure 3, all *Acinetobacter* isolates were resistant to ampicillin and oxacillin, while the rate of ciprofloxacin, imipenem, aztreonam and gentamicin resistance was 68%, 65%, 74% and 69%, respectively. The lowest antibiotic resistance rate was associated with colistin (5%), indicating the high value of this antibiotic for the treatment of infections caused by *Acinetobacter*.

In a study by Dashm et al. in India, of 137 A. baumannii isolates, 74% were MDR and all isolates were susceptible to colistin (14). In the present study, 56% of isolates were MDR and 95% of isolates were sensitive to colistin. In another study in India, 87% of A. baumannii isolates from ICU patients were MDR (15). In a study by Gulbadakh et al. on 75 A. baumannii strains in Turkey, the rate of resistance to ceftazidime, ampicillin, gentamicin and ciprofloxacin was 89%, 97%, 66% and 97.3%, respectively (16).

In our study, of 45 ESBL-positive isolates, the OXA-2 gene was present in 12 cases, the OXA-10 gene was found in five cases and four case contained both genes. In a study by Rahimzadeh et al. in Iran on 60 ESBL-positive isolates, the OXA-2 and OXA-10 genes were present in seven and five isolates, respectively. *P. aeruginosa* is also among the main causes of hospital-acquired infections. Our results showed that *P. aeruginosa* is mostly resistant to cefotaxime and highly susceptible to colistin and tobramycin. In the present study, 24% of the strains were ESBL. According to previous studies, frequency of ESBL genes in *P. aeruginosa* was highest in in China (17) and Iran (18). Of 24 ESBL-positive isolates, six isolates contained the OXA-2 gene, three had the OXA-10 gene and one isolate was positive for both genes. In a study in Iran, 68.7% of ESBL-positive isolates were positive for the OXA-10 gene (18). In a study by Frédéric Bert et al., 26.3% and 4.6% of isolates contained the OXA-10 and OXA-1, respectively (19).

Fluoroquinolones are a group of synthetic antibacterial that are widely used to treat bacterial infections. In the present study, the rate of nalidixic acid and ciprofloxacin resistance in *K. pneumoniae* isolates was 31.5% and 18.4%, respectively. The high rate of nalidixic acid resistance may be because the production of this antibiotic dates back to 1962 (20). In addition, the rate of resistance to second generation fluoroquinolones norfloxacin and ofloxacin was 17.3% and 4.3%, respectively. In line with these results, Madani et al. in Kermanshah (west of Iran) (20), Molaabaszadeh in Tabriz (Northeast) (21) and Kehrenberg in Spain (22) reported the rate of resistance to norfloxacin as 31.3%, 29% and 33%, respectively. In our study, the lowest rate of resistance was related to gatifloxacin, which is a new generation (fourth generation) fluoroquinolone with more potent antibacterial effects than the old fluoroquinolone.

There are three types of plasmid-dependent quinolone resistance genes (23). In the present study, we investigated qnr genes types A, B, C and S and found qnrA, qnrB and qnrS in 17.7%, 48.8% and 8.88% of isolates, respectively. Moreover, we detected simultaneous presence of qnrA and B and qnrB and S in 13% and 11% of the isolates, respectively. However, qnrC was not found in any of the quinolone generators.

In a study by Pakzad et al. (2011), 20.8% of *E. coli* isolates from the Milad Hospital in Tehran were qnrB positive (24). In a study in Khorraramabad (west of Iran), qnrA was detected in 14.3% of *E. coli* isolates (24). Given the increasing prevalence of these genes in *E. coli* strains in different regions of Iran and the possibility of horizontal gene transfer, accurate monitoring of fluoroquinolones administration seems essential. In a similar
study in Jamaica, of 20 K. pneumonia isolates, four strains contained qnrA, six strains carried both qnrA and qnrS, and none contained qnrB or qnrS (25). In another study, of 37 K. pneumoniae isolates, seven (18.92%) isolates contained qnrS, while qnrB was absent (23). The nosocomial infections caused by MRSA and VRE strains are challenging because they are associated with treatment failure and mortality (26, 27). In the present study, the frequency of MRSS and MRSE strains was 24% and 43%, respectively, which are similar to other studies (28). Vancomycin has been commonly used as the antibiotic of choice for the treatment of MRSA infections. Although a high rate of vancomycin resistance has been reported in some studies (27, 29), we observed no such phenomenon in our study. Multiple reports have shown that there is a high prevalence of MDR gram-positive bacteria in the hospital setting (29, 30). Similar to recent reports (3), we detected MDR enterococci isolates from clinical specimens. The most common UTI-related Enterococcus species was E. faecium.

CONCLUSION
Our findings indicate the relatively high frequency of ESBLs, MRSA and VRE strains in clinical isolates from hospitals of Zahedan, southeast of Iran. Horizontal gene transfer and mobile genetic elements can contribute to spread of antibiotic resistance genes in the community and hospitals. In this regard, a global surveillance plan should be coordinated for prevention of this overwhelming increase in emergence of antibiotic resistant bacteria.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

REFERENCES


