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Original Article

Prevalence of Pathogenic Bacteria in Shiraz Hospital Wards and Evaluation of Their Antibiotic Resistance Genes

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Abstract

Background & objectives: Hospital-acquired infections (HAIs) are the most common adverse event in healthcare worldwide. Furthermore, multidrug-resistant organisms are another complication associated with hospital infections. This study aimed to isolate and identify pathogenic bacteria from hospital wards in Shiraz (Namazi and Rajaei) and to evaluate their antibiotic resistance genes.

Materials and Methods: In this cross- sectional study, 50 samples were collected from hospital walls, floors, clothing, beds, endotracheal tubes, and some other areas in Namazi and Rajaei Hospitals between October 2023 to March 2024. The samples were transferred to the laboratory within two hours and cultured on blood agar and Eosin methylene blue agar. The isolates were subjected to determine antibiotic sensitivity using the disk diffusion method to antibiotics viz., gentamicin (30µg), ceftazidime (30µg), chloramphenicol (30µg), tetracycline (30µg), sulfamethoxazole (30µg), and erythromycin (30µg). Molecular identification of the isolates was performed by the 16S rRNA gene, and resistance genes such as *AAC1*, *Ctx-m*, *cmlA1*, *tet(W)*, *Sul1*, and *ermB* were examined using Multiplex PCR.

Results: Of 50 samples, 22 pathogenic bacterial strains were isolated and identified as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Staphylococcus aureus,* and *Escherichia coli.* The results of Antibiotic resistance tests revealed that the highest percentage of resistance was to ceftazidime (89.5%), followed by gentamicin (87.6%), and less resistance was to sulfamethoxazole (26%). The results obtained from the detection of *AAC1*, *Ctx-m*, *cmlA1*, *tet*(W), *Sul1*, and *ermB* showed that *Acinetobacter baumannii* carried all 6 antibiotic resistance genes, while the rest of the pathogenic bacterial isolates carried 4 antibiotic-resistant genes.

Conclusion: Our finding showed that high prevalence of antibiotic-resistant bacteria in hospital environments. Although all isolates exhibited resistance characteristics, *Acinetobacter baumannii* demonstrated the highest level of multidrug resistance.

Keywords: Antibiotic resistance, Pathogenic bacteria, hospital-acquired infections.

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Introduction

Bacteria possess the ability to share their genetic material and transfer resistance genes horizontally. Hospital infections are known as healthcare associated infections (HAIs), are infections that develop during the process of receiving healthcare at various stages. Hospital infections may include occupational infections that can affect staff (1). HAIs are often categorized by the affected body systems, such as lower respiratory tract infections (including bronchitis, tracheobronchitis, bronchiolitis, trachea and lung abscess, or empyema without pneumonia), skin and soft tissue infections, cardiovascular infections, bone and joint infections, central nervous system infections, and genital infections (2). Microbial pathogens responsible for HAIs include bacteria, viruses, and fungi. The prevalence of infections caused by specific microorganisms varies depending on the location and environment of the healthcare facility and the patient population. Generally, bacteria are the most common pathogens, followed by fungi and viruses (3). Hospital acquired infections affect a significant number of patients globally, leading to increased mortality and financial impact on healthcare systems. While the global data of healthcare associated infections is unknown due to a lack of reliable data and surveillance systems (4), healthcare-associated pathogens may have different modes of transmission. The most common mode of transmission is through direct or indirect contact. Common microorganisms that can be transmitted through contact include multidrug-resistant bacteria such as MRSA, ESBL gram-negative organisms, *Clostridium difficile*, and rotavirus. Transmission through respiratory droplets is the second possible route and occurs when microorganisms are spread by large droplets (over 5 microns) (5). Infectious pathogens transmitted through droplets include the novel SARS-CoV-2 virus, *Bordetella pertussis*, and *Neisseria meningitidis*. Airborne transmission involves the transfer of microorganisms from the respiratory tract by small droplets (less than 5 microns) that travel long distances. The chickenpox, tuberculosis, and measles viruses can also be transmitted by the airborne route (6). The aim of this study was investigating the frequency of occurrence of antibiotic resistance in hospital environments and evaluating their antibiotic resistance character. Moreover, the isolates were evaluated for carriage of some antibiotic resistance genes, viz., *AAC1, Ctx-m-32, cmlA1, tet(W), Sul1*, and *ermB*. It must be noted that *Ctx-m-32* gene is related to extended-spectrum beta-lactamase (ESBL) antibiotics, the *ermB* gene is a macrolide-lincosamide-streptogramin B resistance gene, *tet(W)* gene is tetracycline resistance, and *sul1* is a sulphonamide resistance gene (7). Therefore, the present study was conducted to isolate and identify pathogenic bacteria from hospital wards in Shiraz (Namazi and Rajaei) and to evaluate their antibiotic resistance genes.

Materials and methods

A) Sample collection and isolation of pathogenic bacteria: Fifty samples were collected from the environment of Namazi and Rajaei hospitals in Shiraz. The samples were from walls, floors, beds, cloths, endotracheal tubes, etc. Sampling was performed by sterile swabs, and the applied swabs were placed in sterile tubes of physiological serum separately and transferred to the laboratory within two hours. Each sample was cultured on the blood agar, Eosin methylene blue, and mannitol salt agar media. All cultivated plates were

incubated at 37°C for 24-48 hours. After this period, growth colonies with potent pathogenic characters were identified and subjected to determine their antibiotic susceptibility (8).

B) Phenotypic identification: The isolated colonies were phenotypically identified using Gram staining and biochemical tests such as catalase, oxidase, motility, Nitrate, urease, indole, citrate, MR/VP, and triple sugar iron agar.

C) Antibiotic susceptibility test: Antibiotic susceptibility of the isolates was evaluated by the Kirby-Bauer disk diffusion method. To perform the test, Mueller-Hinton agar and the antibiotics gentamicin (30µg), ceftazidime (30µg), chloramphenicol (30µg), tetracycline (30µg), sulfamethoxazole (30µg), and erythromycin (30µg) (Himedia, India) were used. The isolates were cultured on Mueller-Hinton agar, and antibiotic disks were regularly placed on the medium. Then plates were incubated at 37 °C and after 24 to 48 hours, the zone of inhibition around each disk was measured, and susceptible, intermediate and resistance property for each isolate was determined according to CLSI 2021. Tests were conducted in three replicates (9).

D) Molecular identification of antibiotic resistant isolates: All confirmed colonies were extracted for total DNA with QIAamp DNA mini kit (QIAgen, Inc., Valencia, California, USA) according to the manufacturer's instruction. Extracted DNAs were stored at -70ºC until molecular processing. Polymerase Chain Reaction (PCR) was performed in a total volume of 25µl reaction containing: 2.5µl PCR buffer (10x-Qiagene Inc.) 4µl MgCl2 (5.5 mM-Qiagene Inc.), 1µl dNTPs (0.2 mM-Qiagen, Inc.), 1.5U Taq DNA polymerase (Qiagene, Inc.) and 1 µl of each primer (10 pmol/µl) with 3µl of template. Nuclease free sterile double distilled water was added to a final volume of 25µl. The mixture was amplified with the following thermo cycler program: 5 min at 95ºC for initial denaturation, followed by the PCR condition: at 95ºC for 30 sec, 58ºC for 30 sec and 72ºC for 30 sec, for 30 cycles with the 10 min final extension at 72ºC. The PCR products was examined for banding patterns by 8% poly Acryl Amide gel electrophoresis, and visualized by ethidium bromide staining and UV light (8). Universal 16S rRNA primers were used, with the forward primer sequence as AGAGTTTGATCMTGGCTCAG and the reverse primer sequence as CTGCTGCSYCCCGTAG.

E) Detection of antibiotic resistance genes in pathogenic bacteria: Multiplex PCR was applied for detection antibiotic resistance genes of *AAC1*, *Ctx-m-32*, *cmlA1*, *tet(W)*, *Sul1*, and *ermB* genes, thermocycles program was adjusted 5 min at 95ºC for initial denaturation, followed by the PCR condition: at 95ºC for 30 sec, 68ºC for 30 sec and 72ºC for 30 sec, for 40 cycles with the 10-min final extension at 72ºC. Then a total volume of 25µl reaction prepared. This was containing: 2.5µl PCR buffer (10x-Qiagene Inc.) 4µl $MgCl_2$ (5.5mM-Qiagene Inc.), 1µl dNTPs (0.2mM-Qiagen, Inc.), 1.5U Taq DNA polymerase (Qiagene, Inc.) and 1 µl of each primer (10 pmol/µl) with 3µl of template. Nuclease free sterile double distilled water was added to a final volume of 25µl. The PCR products was examined for banding patterns by 8% poly Acryl Amide gel electrophoresis, and visualized by ethidium bromide staining and UV light (Table 1).

F) Electrophoresis: PCR products were examined for banding patterns by agarose gel Electrophoresis with 2% agarose TBE buffer. Three microliters of DNA with a concentration of 10mg/µl were added to each well and PCR

Table 1. Primers for antibiotic resistance genes.

Genes	Primers	Sequence (5' → 3')	PCR Product (bp)	references
aaC1	F R	TTCATCGCGCTTGCTGCYTTYGA GCCACTGCGGGATCGTCRCCRTA	260	15
Ctx-m-32	F R	CGTCACGCTGTTGTTAGGAA CGCTCATCAGCACGATAAAG	156	12
cmlA1	F R	TAGTTGGCGGTACTCCCTTG GAATTGTGCTCGCTGTCGTA	137	16
tet W	F R	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	117	6
Sul1	F R	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	208	15
ermB	F R	AAAACTTACCCGCCATACCA TTTGGCGTGTTTCATTGCTT	103	16

product sample was added into the wells. Apparatus was adjusted at 80 volts for 45 minutes and gel agarose was visualized by ethidium bromide staining and Gel document.

G) Statistical analysis: Statistical analysis was carried out using SPSS21 software. In the first stage, the data were analyzed using the Kolmogorov-Smirnov test, and if the data were not normally distributed, the non-parametric chi-square method with $P \leq 0.05$ was used.

Results

A) Isolation of pathogenic bacteria: The results obtained from the phenotypic identification of the isolates indicated that, out of 50 samples, 22 strains were isolated. As showed in Table 2 these strains were *Pseudomonas aeruginosa, Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus*. High frequency of isolation was related to *Pseudomonas aeruginosa* and less frequency was related to *Stenotrophomonas maltophila* Furthermore, contamination of the trachea samples was relatively more and the wall samples were less.

B) Antibiotic susceptibility test: All isolates had antibiotic-resistant character (Table 3). However, *Acinetobacter baumannii* was relatively more resistant to the tested antibiotics, *Stenotrophomonas maltophila* was relatively more sensitive to them. In addition, in our findings, the best effective antibiotic was Sulfamethoxazole and less effective antibiotic was Ceftazidime.

C) Molecular identification: The results in this part indicated that all antibiotic resistant bacteria were authenticated by molecular method. Based on this method the pathogenic bacteria with resistance character were mentioned in Table 4.

D) Evaluation of antibiotic resistance genes: The results obtained from evolution of existence of *aaC1, Ctx-m-32, cmlA1 tet, W Sul1,* and *ermB* genes in the isolates (Fig. 1) indicated that *Acinetobacter baumannii* possesses the all 6 genes, however, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* possess only four genes. Staphylococcus carriage five genes. All isolates possess *aaC1, tet W* and *erm*B genes. However, only *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were carriage *Ctx-m-32*. Therefore, all isolates have potential for showing resistance character to aminoglycoside, macrolide-lincosamide-streptogramin B and tetracycline antibiotics.

Table 2. Frequency of occurrence of the bacteria in samples of hospitals.

Bacteria	Total (%)	Floor (6)	Wall (7)	Bed (10)	Cloths (7)	ET tube (10)
Pseudomonas aeruginosa	5 (22.70)	1(4.50%)	1(4.50%)	0(0%)	1(4.50%)	1(4.50%)
Stenotrophomonas maltophila	2 (9.10)	0(0%)	0(0%)	0(0%)	0(0%)	2(9.10%)
Acinetobacter baumannii	4 (18.20)	0(0%)	1(4.50%)	0(0%)	2(9.10%)	0(0%)
Klebsiella pneumoniae	4 (18.20)	0(0%)	1(4.50%)	1(4.50%)	1(4.50%)	1(4.50%)
Staphylococcus aureus	4 (18.20)	0(0%)	1(4.50%)	1(4.50%)	2(9.10%)	0(0%)
Escherichia coli	3 (13.60)	0 (0%)	1(4.50%)	1(4.50%)	1(4.50%)	0(0%)
Total	22 (100)	1	5	3	7	4

Table 3. Antibiotic susceptibility of the bacterial isolates.

Bacterial Isolates / Antibiotics	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Acinetobacter baumannii*	*Staphylococcus aureus*	*Stenotrophomonas maltophilia*	*Escherichia coli*
Erythromycin	22±1	12±1	15±2	15±1	22±1	19±1
Gentamicin	15±2	14±2	15±2	16±2	15±2	15±2
Chloramphenicol	10±2	16±2	17±2	16±2	21±2	17±2
Tetracycline	14±2	21±2	19±2	21±2	17±2	23±2
Sulfamethoxazole	19±1	20±1	14±1	19±1	18±1	21±1
Ceftazidime	12±1	14	15±1	13±1	16±1	13±1

Table 4. Molecular identification of the antibiotic resistance bacteria.

Bacterial isolates	% Identification	Accession Number
Staphylococcus aureus	100	CP000255.1
Pseudomonas aeruginosa	100	AF440524.1
Klebsiella pneumonia	97	CP003224.1
Acinetobacter baumannii	100	CP058289.1
Stenotrophomonas maltophilia	100	NR_041577
Escherichia coli	99.70	OM980091

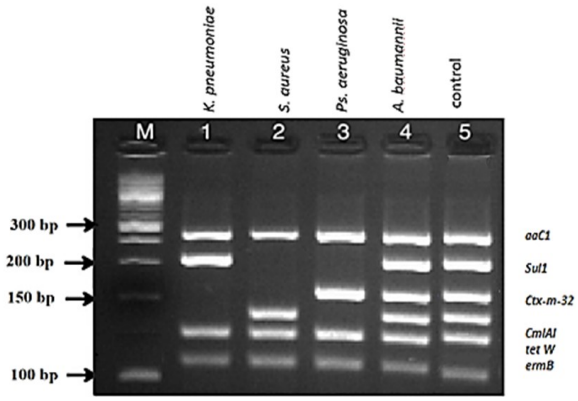

Fig. 1. Gel electrophoresis of *aaC1, Ctx-m-32, cmlA1 tet, W Sul1,* and *ermB* resistance genes in isolates

Discussion

Antibiotic-resistant bacteria are considered one of the most significant problems in today's world, causing various diseases in humans. Therefore, investigating this topic must be considered of utmost importance. Our findings in this study illustrated that the environment of hospitals must also be considered as a source of infection. It is because pathogenic bacteria were isolated from different samples (beds, clothes, floors, walls, and endotracheal tubes) in this study. The pathogenic bacteria were isolated from all samples, however, the endotracheal tubes were relatively more

contaminated. In this regard, Khan *et al*., 2017, reported that four pathogenic bacteria, such as *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* bacteria, were isolated from beds, walls, and floors of hospitals in India (10). Our finding indicated that *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* were isolated from environmental samples in this study. Based on different reports, these bacteria are generally isolated from ICU, CCU and various hospital environments (8). For example, Wagner *et al*., 2014, reported that 750 bacterial species that could be isolated from hospital environments, and generally they had antibiotic resistance characteristics as well as potential for the formation of biofilms (11). The results obtained from antibiotic susceptibility of the isolates indicated that all isolates had antibiotic resistant character. *Acinetobacter baumannii* was relatively more resistant to the tested antibiotics, but *Stenotrophomonas maltophila* was relatively more sensitive. In addition, our finding showed that the best effective antibiotic was Sulfamethoxazole and less effective antibiotic was Ceftazidime. Thus, we continue our research using only antibiotic-resistant isolates. Generally, this idea has been proven that the bacteria with the ability to survive in hospitals can cause hospital-acquired infection, because of their drug-resistant property. Joon Choi *et al*., in 2013 isolated *Acinetobacter baumannii* from different clinical samples and showed that all isolates were often resistant to most antimicrobial agents (12). Almasaudi *et al*., 2018 reported that 121 strains of *Staphylococcus aureus* from hospital samples. They reported that most of them were resistant to common antibiotics such as penicillin, chloramphenicol, tetracycline, sulfamethoxazole, ceftazidime, erythromycin, and gentamicin (13). Tavakoli *et al*., in 2015, isolated *Acinetobacter baumannii* from two hospitals in Tehran. They also confirmed the antibiotic resistance character of this bacterium. They opined that out of 121 *Acinetobacter baumannii* isolates obtained from clinical samples, the highest resistance was observed against antibiotics such as gentamicin, chloramphenicol, tetracycline, sulfamethoxazole, ceftazidime, erythromycin, and gentamicin (14). Shokrzadeh *et al*., in 2017, isolated some strains of *Pseudomonas aeruginosa* from collected samples of burn patients as well as environmental areas. According to their report, the isolates were resistant to the antibiotic tested, even many of strains were MDR and PDR (15). The results obtained from the determination of the frequency of occurrence of *aaC1, Ctx-m-32, cmlA1 tet, W Sul1,* and *ermB* genes in the isolates indicated that *Acinetobacter* strains carried all 6 genes studied, however, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* possessed four genes, and they did not have *Ctx-m-32, cmlA1, sul1,* and *Ctx-m-32 and cmlA1 and Sul1*, respectively. Tangi *et al*., 2015 reported that 43 strains of *Escherichia coli* from hospital samples. The antibiotic resistance of the strains against 10 common antibiotics was evaluated using the simple disk diffusion method. According to the results Gentamicin resistance genes (96.7%) beta-lactam (88.7%), and tetracycline (82.2%) resistance genes were the most prevalent in the bacterial isolates (16). The percentage of resistance to the Drugs used in the present study is almost consistent with the results of this research, and the slight difference is due to the different sources of the strains. In most cases, due to the overuse of antibiotics, we

have had numerous cases of drug resistance in pathogens, which in turn leads to treatment failure and the emergence of many complications despite significant medical expenses. Drug resistance to antibiotics varies in different regions of Iran and the world due to genetic variations in the causative strains, differences in antibiotic consumption rates, and disparities in access to broad-spectrum and new antibiotics (17). Sometimes a microbe needs to be treated with several antibiotics taken simultaneously, and taking a single antibiotic alone or incompletely can lead to resistance. Therefore, determining the antibiotic resistance patterns in common pathogenic bacteria is important for guiding empirical and specific treatments against a particular pathogen.

Conclusion

Horizontal transfer of antibiotic resistance genes among the pathogenic bacteria can cause different problems. In addition, patients, staff, and hospital environments are the sources of pathogenic bacteria with potent antibiotic resistance characteristics. Our finding showed that pathogenic bacteria exist in the hospital wards and most of them carriage antibiotic genes. Among all the isolates, *Acinetobacter baumannii* showed relatively more resistance. Generally, the pathogenic bacteria isolated in this study showed approximately sensitivity to sulfamethoxazole and resistance to ceftazidime.

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Statements and Declarations

Conflicts of interest

The authors declare that there are no conflicts of interest.

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