Identification and Detection of Pathogenic Bacteria From Patients With Hospital Acquired Pneumonia in Southwest of Iran; Evaluation of Biofilm Production and Molecular Typing of Bacterial Isolates

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Research Article

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Abstract

Background

Hospital acquired pneumonia (HAP) HAP is the most second nosocomial infection and the most common infection in the intensive care unit. The aim of the present study was to determine the prevalence of pathogenic bacteria, their biofilm formation and molecular typing in sputum samples from patients with HAP in southwest of Iran.

Methods

In this cross-sectional study, 58 patients with HAP were included in this study. Clinical samples including sputum and endotracheal aspirate collected from each patient under aseptic conditions and were sent to the Microbiology Laboratory. Isolation and detection of bacteria were performed by culture and polymerase chain reaction methods. Biofilm formation was evaluated using Congo red Agar or microtiter plate assay. A rep-PCR typing method was used for typing of gram-negative bacteria. MRSA isolates were typed using SCC*mec* typing method. Data were analyzed by SPSS software version 15 and chi-square test.

Results

Using culture method, bacteria identified in 52 (89.7 %) of patients. *Acinetobacter baumannii* was the most prevalent (37%) organism and followed by *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. All of the *A. baumannii* and *P. aeruginosa* were biofilm producer. 12 cases (80%) of *S. aureus* isolates were MRSA and 91.6% of MRSA were SCC*mec* type III. The rep-PCR analysis showed 7 different patterns in 20 *A. baumannii*, 6 patterns in 13 *P. aeruginosa* and 4 patterns in 6 *E. coli*. Most of the *A. baumannii* isolates were multi drug resistant.

Conclusion

A. baumannii was more prevalent in VAP, while *S. aureus* as a major pathogen in N- VAP that maybe related to tendency of *A. baumannii* to the aquatic environment. Biofilm production ability in bacteria increased antibiotic resistance, development of chronic and persistent infection in HAP. Based on rep-PCR typing method we concluded that bacteria transmitted from patients or health care workers among different wards of hospital. Colistin can be used as a treatment in gram negative MDR isolates.

Background

Hospital acquired pneumonia (HAP) is defined as lung parenchymal infection which does not occur at the time of hospitalization or not during the incubation period but occurs in patients after 48 hours after hospital admission. HAP is the most second nosocomial infection which leads to prolonged hospitalization, increasing costs and also morbidity and mortality[1]. Common signs and symptoms including fever, leukocytosis, purulent secretion, increasing respiratory rate, abnormal chest examination,

tachypnoea and impaired oxygenation[2]. HAP is divided into two subgroups: ventilator-associated pneumonia (VAP) and Non-ventilator Hospital-acquired pneumonia (NV-HAP)[3]. VAP, A subset of hospital-acquired pneumonia that occurred 48 hours or more after tracheal intubation and attachment to the ventilator device, and is the most common infection in the intensive care unit (ICU). NV-HAP occurs in patients who are hospitalized for at least 48 hours and not connected to a ventilator [4, 5]. Bacterial infections is the main cause of HAP which among them *Acinetobacter spp*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (Especially MRSA) were the most common agents [6–8]. The most important sources of these bacteria are the hospital environment, patients microbial flora and primary hospitalized patients[9]. Biofilm formation is one of the most effective factors in the pathogenicity of these bacteria. Biofilm maybe causes antimicrobial resistance in bacteria and as well as interfere with host defense mechanisms. In addition, Biofilm can be as a reservoir for recurrence of these pathogens[10]. The endotracheal tube is a potential reservoir for microorganisms to infect the respiratory tract. The endotracheal tube is a risk factor for ventilator-associated pneumonia because microorganisms can adhere to the surface of the endotracheal tube, and some species form biofilm on the tube. These microorganisms may be transmitted to the lungs during suctioning [11]. Therefore, biofilm producer bacteria can persist in hospital environments and have an important role in nosocomial infections (especially in HAP) and also lead to treatment failure, increasing cost of treatment and mortality in patients.

Molecular typing methods are very important tools in identifying infectious species for epidemiological studies, determining the structure of microbial population and genetic diversity within a species. Molecular typing of bacteria is effective in the prevention and control of infections. REP-PCR is a molecular typing method to determine the clonal relationship, genotyping and determining the phylogenetic relationships of closely related species [12, 13]. This Method is most often referred to as repetitive sequence-based PCR. This technique is a valuable tool for identifying, tracking and studying diversity among microorganisms[14]. *S. aureus* is an important nosocomial pathogen. Many typing methods used for *S. aureus* including: MLST typing, *spa* typing, *SCCmec* typing, and *agr* typing and they are effective methods to describe the epidemiologic trends and implement infection control strategies [15]. The accessory gene regulator (*agr*) is one of the major regulators of virulence factor production (hemolysins, enterotoxins, MSCRAMMs, etc) in *S. aureus* [16]. The *agr* Locus is controlled by P2 and P3 promoters. P2 operon has four genes: *agrA*, *agrB*, *agrC* and *agrD*. The P3 operon transcribes a regulatory RNA molecule named RNAIII[17]. So far four major *agr* groups (I-IV) have been identified based on the amino acid sequence polymorphism of the *agr*-encoded autoinducing peptide (*agrD*) and its corresponding receptor (*AgrC*), (RR). The *agr* types are different in their properties and prevalence in various geographical areas thus; identification of predominant types in each region are useful for epidemiological purpose. Methicillin-resistant strains of *S. aureus* have the *mecA* gene. This gene is located on the staphylococcal SCC chromosome cassette. Based on complete sequence data thirteen *SCCmec* types (I-XIII) have been defined in MRSA[18]. Among the various *SCCmec* types reported worldwide, types I-III are known as predominant hospital-acquired MRSA[19]. *SCCmec* typing is a useful method for detection of strains associated with nosocomial infections [20].

Because Microorganisms responsible for HAP differ between geographical regions and also among hospitals and patients in one region and most of the HAP treated empirically, information on the bacterial pathogen and their antibiotic resistance pattern are necessary. Hence the aims of the present study were to: detection of microorganisms from HAP, evaluation of biofilm production and antimicrobial susceptibility pattern of bacterial isolates and also Molecular typing (SCC*mec* typing, agr typing and REP-PCR) of bacteria isolates, were collected from patients with HAP admitted in ShahidBeheshti and Imam Sajjad hospitals in southwest of Iran.

Method

Sampling Method:

This descriptive cross-sectional study was performed on 58 patients who were hospitalized in Imam Sajjad and ShahidBeheshti hospitals affiliated with Yasuj University of Medical Sciences in southwest Iran from 2018 to 2019. The inclusion criteria as follows: 1) age > 15 years. 2) Having at least one or two of the following characteristics (fever, leukocytosis, leukopenia, purulent secretion of lungs, sputum, and cough). 3) Infection that occurred 48 hours after hospitalization. 4) At least 48 hours after intubation for VAP. Patients unwilling to voluntarily enter the study, those suffering from immunodeficiency and if the patient shows signs of pneumonia within less than 48 hours of hospitalization were excluded. The patients were diagnosed by a physician in internal medicine and infectious diseases. The first morning sputum samples and endotracheal aspirate (ETA) were collected in sterile containers under aseptic conditions and sent to the microbiology laboratory. Samples were divided into two parts. One part was inoculated on McConkey agar, blood agar (5% sheep) and Chocolate agar media, and incubated at 37°C for 24–48 hours. The identification of the bacteria was made based on gram staining, colony morphology, standard biochemical tests and PCR method used for final confirmation of isolated bacteria. Another part stored in -20 for molecular analyses. Demographics data including age, sex and clinical characteristics were collected. Before sampling, informed consent was obtained from each patient. This study has been approved by Yasuj University of Medical Sciences with ethics code (IR.YUMS.REC. 1396. 194.).

Biofilm Formation Assay:

For gram negative bacteria Biofilm formation evaluated using microtiter plate method[21]. First, each isolate was inoculated in Trypticase Soy Broth (TSB) and was incubated at 37°C for 24 h. Then a 1: 100 dilution of this suspension was prepared and 200 µl of this dilution was inoculated in sterile 96- well flat-bottomed microtiter polystyrene plates. The negative control wells only contain sterile TSB medium and incubated for 24 hours at 37 ° C (each test was repeated three times). After this time, the contents of the wells were emptied and each well was gently washed three times with 200 µl of phosphate buffered saline (PBS). The plates were then dried at room temperature, and 150 µl of 99% methanol were added to each well to fix the bacteria inside the wells. After 15 minutes, the contents of the wells were drained and the plates were allowed to dry at laboratory temperature. Then, the wells were stained with 200 µl of 1% crystal violet for 20 minutes. The wells were then rinsed gently with water and 200 µl of acetic acid 33%

was added to each well to remove crystal violet. The plates were then incubated for 15 minutes at 37 ° C and the light absorption of the painted wells at 620 nm was read by ELISA. Based on OD, they were classified into four groups: strong, moderate, weak and non-biofilm producers [22]. For *S. aureus*, Biofilm formation was evaluated by Congo Red Method according to, Avila-Novoa et al method [23]. Black colonies showed a positive result. Weak biofilm producer isolates remained pink and occasional darkening at the centers of colonies was also observed. Bright red colonies were considered negative.

Antimicrobial Susceptibility Testing

Antibiotic susceptibility pattern of isolates was performed by disc diffusion method on Mueller Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guideline. *S. aureus* strain (ATCC 25923) and *E. coli* (ATCC 25922) was used as control strains.

Molecular detection of bacterial pathogens

DNA was extracted from clinical specimens including sputum and endotracheal aspirate by phenol chloroform method and then stored at -20°C as DNA. Also boiling method used for bacteria isolates by culture method in this study. The primers used in this study were shown in Table 1. The PCR program was as follows: PCR protocol was carried out in a thermal cycler (BioRad, T100, USA) with initial denaturation at 94°C for 4 min, followed by 34 cycles of denaturation at 94°C for 45 s, annealing (45 s at 55°C for *nucA* and extension at 72°C for 1 min and was followed by a final cycle of extension for 5 min at 72°C. For detection of *A. baumannii*, *P. aeruginosa*, *E. coli* and *K. pneumoniae* the program as follows: initial denaturation at 94°C for 4 minutes, 30 cycles of denaturation at 94°C for 45 s, annealing (30 s at 59°C for *oprL* and *gltA*, 60 s at 57°C for *ureD* and *aroE* and extension at 72°C for 1 min and was followed by a final cycle of extension for 5 min at 72°C.

Table 1
The primer sequences used in this study.

Target Gene	Primer Sequence (5' →3')	Amplicon Length, bp	Reference
nucA	F - CTG GCA TAT GTA TGG CAA TTG TT R - TAT TGA CCT GAA TCA GCG TTG TCT	670	(24)
aroE	F – AAGGTGCGAATGTGACGGTG R – AACTGGTTCTACGTCAGGCA	620	(25)
Ure-D	F - CCC GTT TTA CCC GGA AGA AG R - GGA AAG AAG ATG GCA TCC TGC	243	(26)
OprL	F – ATGGAAATGCTGAAATTCGGC R – CTTCTTCAGCTCGACGCGACG	504	(27)
mecA	F-GTG AAG ATA TAC CAA GTG ATT R - ATG CGC TAT AGA TTG AAA GGA T	147	(28)
SCC _{med}	F-GCTTTAAAGAGTGTGTTACAGG R-GTTCTCTCATAGTATGACGTCC	613	(28)
SCC _{medI}	F-CGTTGAAGATGATGAAGCG R-CGAAATCAATGGTTAATGGACC	398	
SCC _{medIII}	F-CCATATTGTGTACGATGCG R-CCTTAGTTGTCGTAACAGATCG	280	
SCC _{medVa}	F-GCCTTATTCGAAGAAACCG R-CTACTCTTCTGAAAAGCGTCG	776	
SCC _{medVb}	F-TCTGGAATTACTTCAGCTGC R-AAACAATATTGCTCTCCCTC	493	
SCC _{medVc}	F-ACAATATTTGTATTATCGGAGAGC R-TTGGTATGAGGTATTGCTGG	200	
SCC _{medVd}	F-CTCAAATACGGACCCCAATACA R-TGCTCCAGTAATTGCTAAAG	881	
SCC _{mecV}	F-GAACATTGTTACTTAAATGAGCG R-TGAAAGTTGTACCCTTGACACC	325	

Target Gene	Primer Sequence (5' →3')	Amplicon Length, bp	Reference
agrI	R- GTCACAAGTACTATAAGCTGCGAT	440	(29)
agr II	R- GTATTACTAATTGAAAAGTGCCATAGC	573	
agr III	R- CTGTTGAAAAAGTCAACTAAAAGCTC	406	
agr IV	R- CGATAATGCCGTAATAC CCG	588	
pan-agr	F- ATGCACATGGTGCACATGC		

Molecular typing (SCCmec typing and agr typing)

PCR conditions were as follows: an initial denaturing at 94 °C for 4 min, followed by 35 cycles denaturing at 94 °C for 45 S, annealing (45 s at 60°C for SCC*mec* types I, II, III and V and 45 s at 60.5°C for SCC*mec* subtypes IVa,IVb, IVc and IVd), and extension at 72 °C for 40 S. The final extension was continued at 72 °C for 5 min and were performed in a thermocycler (BioRad, T100, and USA).

Rep-PCR

For Rep-PCR, the primers REP1 (5-GCGCCGICATCAGGC-3) and REP2 (5- ACGTCTTATCAGGCCTAC-3) was used. Amplification reactions were performed in a final volume of 25 µL, containing 12.5 µl of Master Mix(Amplicon, Denmark), 25 pmolof each primer, 5 µl of bacterial DNA. The PCR protocol included an initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 1 min, and final extension of 16 min at 72°C. The PCR products were electrophoresed on 2% agarose gel at 90 V for 45 min (for rep- PCR 60 V for 150 min), stained with DNA safe Stain and visualizedunder UV light using gel documentation system (Major Scienses, Taiwan).

Results

Totally 58 patients participated in the study. At least one bacterial isolates identified in 52 (89.7 %)of patients using culture method. In culture positive patients: the average age of 57.92 ± 18.59 (range between: 18–94) years and 52% of the patients were female.Thirty-six (69%) patients were VAP andsixteen(31%) patients were NV-HAP. The most common clinical symptoms and laboratory results were leukocytosis (100%), fever (82.7%) and sputum (82.7%). The demographic and clinical characteristics of patients with VAP and NV HAP showed in Table 2.

Table 2
Characteristics of patients with HAP.

	culture positive			culture negative
	Total (N = 52)	VAP (N = 36)	NV HAP (N = 16)	Total (N = 6)
Sex				
Female	27 (51.9%)	17 (47.2%)	10 (62.5%)	2(33.3%)
Man	25 (48.1%)	19 (52.8%)	6 (37.5%)	4(66.7%)
Average age	57/92	59/39	54/64	62/5
Clinical signs				
Fever	43 (82.7%)	30 (83.3%)	13 (81.2%)	5(83.3%)
Sputum	43 (82.7%)	30 (83.3%)	13 (81.2%)	4(66.7%)
Chest pain	15 (28.8%)	9 (25%)	6 (37.5%)	2(33.3%)
Cough	10 (19.2%)	7 (19.4%)	3 (18.7%)	2(33.3%)
Underlying disease	20 (38.4%)	14(38.9%)	6 (37.5%)	1(16.7%)

Detection of bacteria by culture and PCR method:

Using culture method 54 bacterial isolates were collected from 52 patients, which among them gram-negative bacilli obtained in 39 (72.2%) and gram-positive cocci (*S. aureus*) in 15 (27.8%). Among gram negative bacteria *A. baumannii* in 20 (37%), *P. aeruginosa* in 13 (24.1%) and *E. coli* in 6 (11.1%). Most of bacterial isolates from patients with VAP were gram negative(84.6%),while in NV-HAP gram positive bacteria (*S. aureus*) 56.3% was prominent. Totally *A. baumannii* was the most predominant isolates identified in 37% of patients. *A. baumannii* identified more in VAP than NV-HAP and it was statistically significant ($P = 0.016$). Also *S. aureus* detected more in NV-HAP than VAP and it was statistically significant ($P = 0.003$). (More details were shown in the Table 3).

Table 3
Bacterial isolated in this study from patients with VAP and NV-HAP.

Bacteria	VAP		NV-HAP		P-Value*	P-Value**
	Culture (N = 38)	PCR (N = 39)	Culture (N = 16)	PCR (N = 17)		
<i>A. baumannii</i>	18 (47.4%)	18 (46.2%)	2 (12.5%)	2 (11.8%)	0.016	0.014
<i>P. aeruginosa</i>	10 (26.3%)	10 (25.6%)	3 (18.7%)	3 (17.6%)	0.554	0.518
<i>S. aureus</i>	6 (15.8%)	6 (15.4%)	9 (56.3%)	9 (52.9%)	0.003	0.004
<i>E. coli</i>	4 (10.5%)	5 (12.8%)	2 (12.5%)	2 (11.8%)	0.832	0.918
<i>k.pneumoniae</i>	0	0	0	1 (5.9%)	-	-
<i>Total</i>	100%	100%	100%	100%		
*Between the cultures of the two groups VAP and NV-HAP.						
** Between the PCR of the two groups VAP and NV-HAP.						

Using PCR method 56 bacteria were detected which 69.6% were detected from patients with VAP and 30.4% from NV-HAP patients. The detection rate of microorganisms were as follow: *A. baumannii* 35.7% (n = 20), *S. aureus* 26.8% (n = 15), *P. aeruginosa* 23.2% (n = 13), *E. coli* 12.5% (n = 7) and *K. pneumoniae* 1.8% (n = 1). There were no significant differences between bacterial isolated by culture and PCR method.

Biofilm production:

Totally 94.4% of isolates were biofilm producer. Among the isolates, the highest rate of biofilm formation was observed in *A. baumannii* and *P. aeruginosa* (100%), and followed by *S. aureus* (86.7%) and *E. coli* (83.4%). Table 4.

Table 4
Biofilm production of bacterial isolates in the present study.

	microtiter plate method			Congo Red Method	Total (N = 54)
	<i>A. baumannii</i> (N = 20)	<i>P. aeruginosa</i> (N = 13)	<i>E. coli</i> (N = 6)	<i>S. aureus</i> (N = 15)	
Strong biofilm producer	18 (90%)	12 (92.3%)	3 (50%)	7 (46.7%)	40 (74%)
Moderate biofilm producer	2 (10%)	1 (7.7%)	1 (16.7%)	4 (26.7%)	8 (14.8%)
Weak biofilm producer	-	-	1 (16.7%)	2 (13.3%)	3 (5.6%)
Negative biofilm	-	-	1 (16.6%)	2 (13.3%)	3 (5.6%)

SCC mec typing:

The *mecA* gene was found in 12 (80%) of *S. aureus* isolates and defined as MRSA which among them 11 cases (91.6%) harbored SCCmec type III (was the most frequent SCCmec type) and 1 case (8.4%) harbored SCCmec type I. The types II, IVa, IVb, IVc, IVd, and V were not recognized in any isolates.

Agr typing:

Among 15 *S. aureus* isolates, 73% (n = 11) were successfully typed by agr typing method. The agr type III was the most prevalent agr type identified in (54%, n = 6) and followed by agr type I (36%, n = 4) and agr type II (9%, n = 1). No agr types IV were observed in isolates.

REP-PCR typing:

Overall in gram-negative bacteria the rep-PCR typing pattern of isolates (based on band pattern similarity) showed great genetic diversity among isolates. The rep-PCR analysis showed 7 different patterns in 20 *A. baumannii*, 6 patterns in 13 *P. aeruginosa* and 4 patterns in 6 *E. coli*. (Table 5 and Fig. 1).

Table 5
Typing of gram negative bacteria based on rep-PCR typing method.

Bacterial isolated	A	B	C	D	E	F	G
<i>A. baumannii</i>	1,2,12,4,14,19	4,6,8,9,13,15	3,10,17	5,11	16,18	7	12
<i>P. aeruginosa</i>	2,5,11	3,6	4,9	7	8	10	-
<i>E. coli</i>	1,2	3,5	4	7	-	-	-

Antibiogram:

Highest rate of resistance were observed among gram negative bacteria to Levofloxacin, Cefotaxime and ceftriaxone. *S. aureus* isolates have high resistance rate to Levofloxacin, and penicillin. Table 6 presents the antibiotic resistance patterns of the Gram-positive and Gram-negative bacterial isolates.

Table 6
The antibiotic resistance patterns of the Gram-positive and Gram-negative bacterial isolates.

Antibiotics	<i>S. aureus</i>	<i>A. baumannii</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
Penicillin	93%	-	-	-
Cefoxitin	80%	-	-	-
Clarithromycin	73%	-	-	-
Sulfamethoxazole	60%	-	-	-
Vancomycin	20%	-	-	-
Clindamycin	73%	-	-	-
Levofloxacin	80%	100%	83.3%	71.4%
Colistin	-	15%	0%	7.7%
Cefepime	-	38.1%	66.7%	35.7%
Ceftriaxone	-	100%	66.7%	78.6%
Meropenem	-	95.2%	33.3%	64.3%
Cefotaxime	-	100%	66.7%	85.7%
Azithromycin	80%	-	-	-
Piperacillin + Tazobactam		100%	33.3%	64.3%

Discussion

HAP is a common, serious and costly problem in hospitals, it is a dangerous complication and an important cause of death in patients admitted to the intensive care unit (ICU) [30–32]. The rate of HAP occurs 21 cases per 1,000 hospital admissions [33]. In various studies, gram-negative bacteria are more prevalent in HAP than gram-positive bacteria [34–36]. In the present study, Gram-negative bacteria (*A. baumannii*, *P. aeruginosa*, *E. coli* and *K. pneumoniae*) were isolated in 72.2%, which was in accordance with previous studies such as Bozorgmehr et al and Boostani et al. from Iran and Jones et al. from (USA) [35–37]. The frequency HAP causing bacteria may vary by hospital, geographic area, patient status, patient's immune system, and health status. *A. baumannii* is an opportunistic pathogen associated with nosocomial infections, especially in ICU. In the current study, *A. baumannii* Similar to previous studies [37–40] was the most common microorganism isolated from HAP which showed the importance of *A.*

baumannii in HAP. The high percentage of *A. baumannii* isolates in this study indicated the function of this bacterium in ability to colonize (in the hospital environment, staff hands and patient's equipment), biofilm formation, transmission of the organism to the lower airways during suctioning in ventilated patients and also the presence of patients colonized with *A.baumannii*.

In the present study *S.aureus* was the second organism in this study (27.8%). It was similar to the study by Ronald et al.[41] and was higher than the values reported in other studies [34, 42, 43]. The presence of *S. aureus* in hospital environment and colonization in long period may be related with (microbial surface component recognizing adhesive matrix molecules (MSCRAMM), biofilm formation, teichoic acid. *Paeruginosa* was the third (24%) isolated pathogen in the present study. *Paeruginosa* is one of the most common opportunistic pathogens and the cause of serious nosocomial infections. It is a stable pathogen that easily forms biofilms and colonizes the body. *A. baumannii* and *P. aeruginosa* were the most common isolated organisms in VAP. This may be due to the tendency of *P. aeruginosa* and *A. baumannii* to the aquatic environment. *E. coli* was the fourth organism in this study (11.2%), was higher than the earlier reported [39, 41, 44], while *K.pneumoniae* was not isolated in any case by culture method in this study unlike many earlier studies [39, 42, 44]. The development of nosocomial infections in hospitalized patients (especially in the ICU) is usually associated with the use of devices such as ventilators, venous catheters, and urinary catheters. Many infections caused by these devices are related to contamination by biofilm-causing microorganisms. In general, colonization and biofilm are two important factors in the HAP causing bacteria [10]. Endotracheal tube, being a potential source of contamination for the lungs of the intubated patients, in one way it can cause damage and transfer bacteria from the pharyngeal cavity to the lower airways[45] on the other hand, bacteria can form a biofilm on it, which is removed during the suction process and goes into the lower device [46]. In addition, biofilm and colonization can establish microorganisms in the hospital environment for longer times. In the present study, 94% of the isolates were able to form biofilm, which was in consistent with Gil-Perotin study[10]. In the present study all of the *A. Baumannii* and *P. aeruginosa* isolates were biofilm producer which is similar to the studies by Asadian et al [47] and Alonso et al [48], that indicates the important role of biofilm in HAP causing bacteria. The biofilm formation in bacteria can lead to increased antibiotic resistance, development of chronic and persistent infection and also increase mortality in patients affected with HAP.

The rep-PCR analysis showed 7 different patterns in 20 *A. baumannii*, 6 patterns in 13 *P. aeruginosa* and 4 patterns in 6 *E. coli* which showed a high diversity of patterns among bacterial isolates which may be related to the transmission of bacteria from patients or health care workers among different wards of hospital. Based on the analysis on rep-PCR typing method in gram-negative bacteria we concluded that bacterial isolates with similar patterns are isolated from patients at consecutive intervals which may be caused by a common source of infection.

In the present study 91.6% of MRSA isolates harbored SCCmec type III which in similarity to our finding SCCmec type III was detected in most nosocomial MRSA strains in other countries[49–51]. The predominance of SCCmec type III, showed the hospital origin of *S. aureus* isolates in the present study,

hence the infection control program is necessary to limiting or eradicating the circulated bacteria in hospital.

Highest rate of resistance were observed among gram negative bacteria to Levofloxacin, Cefotaxime and ceftriaxone. Most of the *A. baumannii* in this study were MDR. Highest rate of antibiotic resistance were observed to Levofloxacin, Ceftriaxone, Meropenem, Cefotaxim and Piperacillin + Tazobactam, which in accordance with other studies that showed most of the *A. baumannii* were MDR [44, 52, 53]. More than 60% of *P. aeruginosa* isolates were resistant to Levofloxacin, Ceftriaxone, Meropenem, Cefotaxime, Piperacillin + Tazobactam, in contrast with this study, Delle Rose et al from Italy [54] reported that only 33.3% of *P. aeruginosa* were resistant to Meropenem. Also, in Delle Rose et al study 31.2%, 25% and 45% of isolates were resistant to Levofloxacin, Ceftriaxone and Tazobactam respectively which is lower than our study, while in another study from India by Tiwari et al [44] 80% of their isolates showed resistant to Meropenem. Differences in antibiotics resistance in different studies may be related to health care policy in different countries, length of hospital stay, geographical distribution of bacterial isolates. Most of the gram-negative bacteria isolated in the present study were sensitive to colistin, which can be used as a treatment in gram negative MDR isolates. According to the results of the present study, the rate of antibiotic resistance was increasing dramatically, and maybe related to the excessive use of antibiotics, empirical therapy without antibiogram results, lack of proper sterilization of ventilation devices after each use and use of non-standard ventilation systems.

Conclusion

The most common isolated bacteria in HAP were *A. baumannii*, *S. aureus*, *P. aeruginosa* and *E. coli*, which among them *A. baumannii* was more prevalent in VAP, while *S. aureus* as a major pathogen in N- VAP, that maybe related to tendency of *A. baumannii* to the aquatic environment. Most of the bacterial isolates were biofilm producer which can lead to increased antibiotic resistance, development of chronic and persistent infection in HAP. Most of the gram-negative bacteria isolated in the present study were sensitive to colistin, which can be used as a treatment in gram negative MDR isolates. Based on rep-PCR typing method, high diversity of patterns were observed among bacterial isolates which may be related to the transmission of bacteria from patients or health care workers among different wards of hospital.

Declarations

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Authors' contributions: SS Kh, FM and SH assisted with data analysis, interpretation, and drafting of the manuscript. SSKh, FM, NR and SH assisted with study design, statistical analysis and drafting of the manuscript. NR assisted with statistical analysis of the manuscript. GN, FM and SH contributed to measuring samples in this study. SSKh, SH, ASH and FM contributed to data interpretation and critically reviewed the manuscript. All authors planned the study design, contributed to the interpretation of the

data, drafted and approved the submitted manuscript. **Funding:** This study has been supported by Deputy of Research and Technology, Yasuj University of Medical Sciences

Ethics approval and consent to participate

This protocol was approved by Yasuj University of Medical Sciences, Ethics Committee with Ethical code number: IR.YUMS.REC. 1396. 194. Prior to sample collection, written informed consents were obtained from of each individual.

Consent for publication

Not applicable.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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References

1. Koulenti D, Tsigou E, Rello J. Nosocomial pneumonia in 27 ICUs in Europe: perspectives from the EU-VAP/CAP study. *European journal of clinical microbiology & infectious diseases*. 2017;36(11):1999-2006.
2. Andrews CP, Coalson JJ, Smith JD, et al. Diagnosis of nosocomial bacterial pneumonia in acute, diffuse lung injury. *Chest*. 1981;80(3):254-8.
3. James Davis B, Finley E, Authority PPS. *The Breadth of Hospital-Acquired Pneumonia: Nonventilated versus Ventilated Patients in Pennsylvania*. 2012.

4. Kalil AC, Metersky ML, Klompas M, et al. Management of adults with hospital-acquired and ventilator-associated pneumonia: 2016 clinical practice guidelines by the Infectious Diseases Society of America and the American Thoracic Society. *Clinical Infectious Diseases*. 2016;63(5):e61-e111.
5. Giuliano KK, Baker D, Quinn B. The epidemiology of nonventilator hospital-acquired pneumonia in the United States. *American journal of infection control*. 2018;46(3):322-7.
6. Chou C-C, Shen C-F, Chen S-J, et al. Recommendations and guidelines for the treatment of pneumonia in Taiwan. *Journal of Microbiology, Immunology and Infection*. 2019;52(1):172-99.
7. Leone M, Bouadma L, Bouhemad B, et al. Hospital-acquired pneumonia in ICU. *Anaesthesia Critical Care & Pain Medicine*. 2018;37(1):83-98.
8. Weber DJ, Rutala WA, Sickbert-Bennett EE, et al. Microbiology of ventilator-associated pneumonia compared with that of hospital-acquired pneumonia. *Infection Control & Hospital Epidemiology*. 2007;28(7):825-31.
9. Hanulík V, Uvízl R, Husíčková V, et al. Bakteriální původci pneumonií u pacientů v intenzivní péči. *Klin Mikrobiol Inf Léč*. 2011;17(4):135-40.
10. Gil-Perotin S, Ramirez P, Marti V, et al. Implications of endotracheal tube biofilm in ventilator-associated pneumonia response: a state of concept. *Critical care (London, England)*. 2012;16(3):R93.
11. Adair CG, Gorman SP, Feron BM, et al. Implications of endotracheal tube biofilm for ventilator-associated pneumonia. *Intensive care medicine*. 1999;25(10):1072-6.
12. Meshkat Z, Salimizand H, Amini Y, et al. Molecular characterization and genetic relatedness of clinically *Acinetobacter baumannii* isolates conferring increased resistance to the first and second generations of tetracyclines in Iran. *Annals of Clinical Microbiology and Antimicrobials*. 2017;16(1):51.
13. Nowak J, Zander E, Stefanik D, et al. High incidence of pandrug-resistant *Acinetobacter baumannii* isolates collected from patients with ventilator-associated pneumonia in Greece, Italy and Spain as part of the MagicBullet clinical trial. *Journal of Antimicrobial Chemotherapy*. 2017;72(12):3277-82.
14. Ishii S, Sadowsky MJ. Applications of the rep-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution. *Environmental Microbiology*. 2009;11(4):733-40.
15. Han L-Z, Ho P-L, Ni Y-X, et al. Panton-Valentine Leukocidin-Positive MRSA, Shanghai. *Emerg Infect Dis*. 2010;16(4):731.
16. Yarwood JM, Schlievert PM. Quorum sensing in *Staphylococcus* infections. *The Journal of clinical investigation*. 2003;112(11):1620-5.

17. Cafiso V, Bertuccio T, Santagati M, et al. agr-Genotyping and transcriptional analysis of biofilm-producing *Staphylococcus aureus*. *FEMS immunology and medical microbiology*. 2007;51(1):220-7.
18. Singh-Moodley A, Lowe M, Mogokotleng R, et al. Diversity of SCC mec elements and spa types in South African *Staphylococcus aureus* mec A-positive blood culture isolates. *BMC Infectious Diseases*. 2020;20(1):1-12.
19. Lim K, Yeo CC, Suhaili Z, et al. Comparison of Methicillin-Resistant and Methicillin-Sensitive *Staphylococcus aureus* Strains Isolated from a Tertiary Hospital in Terengganu, Malaysia. *Jpn J Infect Dis*. 2012;65:502-9.
20. Wang JL, Wang JT, Chen SY, et al. Distribution of Staphylococcal cassette chromosome mec Types and correlation with comorbidity and infection type in patients with MRSA bacteremia. *PloS one*. 2010;5(3):e9489.
21. Jabalameli F, Mirsalehian A, Khoramian B, et al. Evaluation of biofilm production and characterization of genes encoding type III secretion system among *Pseudomonas aeruginosa* isolated from burn patients. *Burns*. 2012;38(8):1192-7.
22. Stepanović S, Vuković D, Hola V, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis*. 2007;115(8):891-9.
23. Avila-Novoa M-G, Iñíguez-Moreno M, Solís-Velázquez O-A, et al. Biofilm Formation by *Staphylococcus aureus* Isolated from Food Contact Surfaces in the Dairy Industry of Jalisco, Mexico. *Journal of Food Quality*. 2018;2018:1746139.
24. Askarinia M, Ghaedi M, Manzouri L, et al. The Effect of Cu-BPDCA-Ty on Antibacterial Activity and The Expression of mecA Gene in Clinical and Standard Strains of Methicillin-Resistant *Staphylococcus aureus*. *Jundishapur J Microbiol*. 2018;11(3):e60680.
25. Noller AC, McEllistrem MC, Stine OC, et al. Multilocus Sequence Typing Reveals a Lack of Diversity among *Escherichia coli* O157:H7 Isolates That Are Distinct by Pulsed-Field Gel Electrophoresis. *J Clin Microbiol*. 2003;41(2):675.
26. Zamani A, Yousefi Mashouf R, Ebrahimzadeh Namvar AM, et al. Detection of magA Gene in *Klebsiella* spp. Isolated from Clinical Samples. *Detection of magA*. *Iran J Basic Med Sci*. 2013;16(2):173-6.
27. Najafimosleh M, Rashnotaie S, Ghaznavi Rad E, et al. Designing of the specific DNA primers for detection of the *exoA*, *oprL* and *algD* pathogenicity genes for rapid diagnosis of *Pseudomonas aeruginosa*. *Tehran-Univ-Med-J*. 2013;71(8):493-501.
28. Zhang K, McClure JA, Elsayed S, et al. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome mec types I to V in methicillin-resistant

Staphylococcus aureus. J Clin Microbiol. 2005;43(10):5026-33.

29. Shopsin B, Mathema B, Alcabes P, et al. Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. J Clin Microbiol. 2003;41(1):456-9.
30. Torres A, Niederman MS, Chastre J, et al. International ERS/ESICM/ESCMID/ALAT guidelines for the management of hospital-acquired pneumonia and ventilator-associated pneumonia: guidelines for the management of hospital-acquired pneumonia (HAP)/ventilator-associated pneumonia (VAP) of the European Respiratory Society (ERS), European Society of Intensive Care Medicine (ESICM), European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and Asociación Latinoamericana del Tórax (ALAT). European Respiratory Journal. 2017;50(3).
31. Kollef MH, Hamilton CW, Ernst FR. Economic impact of ventilator-associated pneumonia in a large matched cohort. 2012.
32. Iwata K, Igarashi W, Honjo M, et al. Hospital-acquired pneumonia in Japan may have a better mortality profile than HAP in the United States: a retrospective study. Journal of Infection and Chemotherapy. 2012;18(5):734-40.
33. Chawla R. Epidemiology, etiology, and diagnosis of hospital-acquired pneumonia and ventilator-associated pneumonia in Asian countries. American Journal of Infection Control. 2008;36(4, Supplement):S93-S100.
34. Farid GA, Moghaddam AB, Bojdy A. Nosocomial pneumonia in patients admitted to the intensive care unit of a tertiary care center in Mashhad, northeast of Iran; an etiologic survey. Archives of Clinical Infectious Diseases. 2018;13(4).
35. Djordjevic ZM, Folic MM, Jankovic SM. Distribution and antibiotic susceptibility of pathogens isolated from adults with hospital-acquired and ventilator-associated pneumonia in intensive care unit. Journal of infection and public health. 2017;10(6):740-4.
36. Boostani V, Dehghan F, Karmostaji A, et al. Incidence of hospital-acquired bacterial pneumonia and its resistance profiles in patients admitted to intensive care unit. Global Journal of Health Science;. 2017.
37. Japoni A, Vazin A, Davarpanah MA, et al. Ventilator-associated pneumonia in Iranian intensive care units. The Journal of Infection in Developing Countries. 2011;5(04):286-93.
38. Salehifar E, Abedi S, Mirzaei E, et al. Profile of Microorganisms Involved in Nosocomial Pneumonia and Their Antimicrobial Resistance Pattern in Intensive Care Units of Imam Khomeini Hospital, Sari, 2011-2012. Journal of Mazandaran University of Medical Sciences (JMUMS). 2013.
39. Bozorgmehr R, Bahrani V, Fatemi A. Ventilator-associated pneumonia and its responsible germs; an epidemiological study. Emergency. 2017;5(1).

40. Werarak P, Kiratisin P, Thamlikitkul V. Hospital-acquired pneumonia and ventilator-associated pneumonia in adults at Siriraj Hospital: etiology, clinical outcomes, and impact of antimicrobial resistance. *J Med Assoc Thai*. 2010;93(Suppl 1):S126-38.
41. Jones RN. Microbial etiologies of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia. *Clinical infectious diseases*. 2010;51(Supplement_1):S81-S7.
42. Yin Y, Zhao C, Li H, et al. Clinical and microbiological characteristics of adults with hospital-acquired pneumonia: a 10-year prospective observational study in China. *European Journal of Clinical Microbiology & Infectious Diseases*. 2020:1-8.
43. Naidus EL, Lasalvia MT, Marcantonio ER, et al. The Diagnostic Yield of Non-Invasive Microbiologic Sputum Sampling in a Cohort of Patients with Clinically Diagnosed Hospital-Acquired Pneumonia. *Journal of hospital medicine*. 2018;13(1):34.
44. Tiwari U, Loomba P, Dogra V, et al. Bacteriological Profile Of Nosocomial Pneumonia Patients In A Superspeciality Hospital. *National Journal of Integrated Research in Medicine*. 2016;7(3).
45. Rello J, Sonora R, Jubert P, et al. Pneumonia in intubated patients: role of respiratory airway care. *American journal of respiratory and critical care medicine*. 1996;154(1):111-5.
46. Sottile FD, Marrie TJ, Prough DS, et al. Nosocomial pulmonary infection: possible etiologic significance of bacterial adhesion to endotracheal tubes. *Critical care medicine*. 1986;14(4):265-70.
47. Asadian M, Azimi L, Alinejad F, et al. Molecular characterization of *Acinetobacter baumannii* isolated from ventilator-associated pneumonia and burn wound colonization by random amplified polymorphic DNA polymerase chain reaction and the relationship between antibiotic susceptibility and biofilm production. *Advanced biomedical research*. 2019;8.
48. Alonso B, Fernández-Barat L, Di Domenico EG, et al. Characterization of the virulence of *Pseudomonas aeruginosa* strains causing ventilator-associated pneumonia. *BMC infectious diseases*. 2020;20(1):1-8.
49. Kilic A, Guclu AU, Senses Z, et al. Staphylococcal cassette chromosome mec (SCC mec) characterization and panton-valentine leukocidin gene occurrence for methicillin-resistant *Staphylococcus aureus* in Turkey, from 2003 to 2006. *Antonie Van Leeuwenhoek*. 2008;94(4):607-14.
50. Lulitanond A, Chanawong A, Sribenjalux P, et al. Preliminary report of SCCmec-types and antimicrobial susceptibilities of methicillin-resistant *Staphylococcus aureus* isolates from a university hospital in Thailand. *Southeast Asian journal of tropical medicine and public health*. 2010;41(4):920.
51. Peng Q, Hou B, Zhou S, et al. Staphylococcal cassette chromosome mec (SCCmec) analysis and antimicrobial susceptibility profiles of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates in a teaching hospital, Shantou, China. *African Journal of Microbiology Research*. 2010;4(9):844-8.

52. ALIKIAI B, AGHADAVOUDI O, EMAMI N. Evaluating antibiotic resistance pattern of ventilator-associated pneumonia in intensive care units of Alzahra Hospital, Isfahan University of Medical Sciences, Iran. 2016.
53. Joseph NM, Sistla S, Dutta TK, et al. Ventilator-associated pneumonia in a tertiary care hospital in India: role of multi-drug resistant pathogens. *The Journal of Infection in Developing Countries*. 2010;4(04):218-25.
54. Delle Rose D, Pezzotti P, Fortunato E, et al. Clinical predictors and microbiology of ventilator-associated pneumonia in the intensive care unit: a retrospective analysis in six Italian hospitals. *European Journal of Clinical Microbiology & Infectious Diseases*. 2016;35(9):1531-9.

Figures

Figure 1

Molecular typing of gram negative isolates using rep-PCR method. A ; showed different patterns in *A. baumannii*, B; showed different patterns in *P. aeruginosa*.