Original Article

Phenotypic and molecular study of beta-lactam resistance in coagulase-negative staphylococci samples

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Abstract

Introduction:

The emergence of resistance to beta-lactam antibiotics is one of the most common resistance cases in Staphylococcus, including coagulase-negative Staphylococcus in hospital pathogens. Therefore, detection of resistance factors can be helpful in treatment process. This study aimed to determine betalactam resistance in coagulase-negative Staphylococcus by phenotypic and genotypic methods.

Materials and Methods:

Totally, 710 isolates were collected from clinical samples in health centers of Zahedan. After genus and species of isolates were determined, their sensitivity to 10 beta-lactam antibiotics was measured via disc diffusion method. PCR and specific primers were used to trace blaZ and mecA gens.

Results:

Of the total samples, 79 isolates were Staphylococcus saprophyticus and 198 isolates were Staphylococcus epidermidis. The resistance profile of the Staphylococcus saprophyticus isolates is as follows: 74 isolates (98.1%) to penicillin, 69 isolates (34.87%) Oxacillin, 31 isolates (24.39%) to ceftriaxone, 71 isolates (87.89%) to Cefoxitin, 43 isolates (43.54%) to cefotaxime, 19 isolates (24.50%) to cefazolin and 27 isolates (17.34%) to cephalexin. PCR results confirmed the presence of mecA and blaZ in most isolates.

Conclusion:

Given the frequency of mecA and blaZ genes and phenotypic test results in coagulase negative Staphylococcus, resistant to different groups of antibiotics is on the rise.

Keywords: Coagulase-Negative, Beta-Lactam, Antibiotic Resistance, Methicillin Resistance

Introduction

Coagulase-negative staphylococci (CoNs) are one of the key pathogens in nosocomial catheter-associated infections (1). These bacteria possess special adhesive properties that help organisms attach to and colonize in devices (2). They live as the normal flora on human skin, and become pathogenic and cause infection under certain conditions (3). Coagulase-negatives are responsible for extensive infections in humans and livestock, and can spread several

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infections in hospitals and the community (4). Two of the most important coagulase-negative staphylococci are staphylococcus saprophyticus and staphylococcus epidermidis (5). These bacteria that have been regarded as saprophytes for many decades have become invasive and pathogenic due to increased use of medical devices such as catheters, cutters, and prostheses (6). This has made this group of bacteria, especially those named above, to be classified as bacteria responsible for nosocomial infections (7). Infections with coagulase-negative staphylococci are often treated with beta-lactam antibiotics, in which, the new generations of cephalosporin have the greater share (8). However, treatment of infections related to this organism with beta-lactam antibiotics has failed due to the rapid increase in resistance to them (9). The main mechanisms of staphylococci resistance to beta-lactam antibiotics are divided into two groups (10). The first is disablement of penicillin mediated by hydrolysis of beta-lactam ring. The second, which is more frequently seen in humans, is synthesis of beta-lactam and Penicillin Binding Proteins (PBPs), which diminishes the affinity for medication and leads to extensive resistance to semi-synthetic penicillins, cephalosporins and carbapenems (11). Other varieties of PBPs include PBP4 and PBP2A, and because of the importance of PBP2 in causing methicillin resistance, the focus is rather on this protein (12). Beta-lactamases selectively open beta-lactam ring, so that the altered structure of this medicine cannot effectively bond with PBPs, and as a result, synthesis of the cell wall continues (13). In addition to blaZ, presence of mecA gene can also cause the incidence of resistance to a wide range of beta-lactams including methicillin, and thus facilitate emergence of methicillin-resistant strains (1). Secretion of Beta-lactamases causes bacterial resistance to beta-lactam antibiotics, and furthermore causes hypersecretion in some strains that lack methicillin-resistant genes, leading to the incidence of false (negative) resistance to methicillin (14). Studies conducted on blaZ reveal that this gene is a signal converter that encodes penicillin-resistance and contains suppressive structural parts, which are encoded by various blaZ groups (15). Resistance created in coagulase-negative group is observed in humans and animals alike, where in some cases, they are transmitted through meat and milk (16 and 17). Identifying these pathogens in causing nosocomial infections is highly important; but it is often performed using methods that are associated with errors or lack sufficient sensitivity (18). The present study was conducted with the aim to determine antibiotic sensitivity pattern in clinical samples of coagulase-negative staphylococci to beta-lactam antibiotics, and to ensure the presence of mecA and blaZ genes.

Materials and Methods

The present descriptive cross-sectional study was conducted between December 2014 and August 2015 on 710 clinical coagulase-negative samples, selected by convenient sampling and collected from health centers in the city of Zahedan. Bacterial nature of samples was considered as study inclusion criterion. Samples included urine, blood, wound discharges, phlegm, tracheal tube, and chest tube, and catheter tip. Staphylococci isolates were identified in-vitro using gram-staining, and standard biochemical tests such as catalase, coagulase, mannitol fermentation, and DNase (medium: Sigma-America). Staphylococcus saprophyticus was identified and differentiated from staphylococcus epidermidis using Novobiocin discs and Nalidixic acid (MAST-England) as well as PYR test, ornithine decarboxylation test, and acid production from maltose, trehalose, mannitol, sucrose, and anaerobic non-fermented glucose. In the disc diffusion technique, first, a suspension is prepared from 12 or 24-hour bacterial culture with

opacity of 0.5 McFarland. This suspension is cultured on a 5mm thick Mueller Hinton medium (Merck-Germany). Discs are placed onto the medium with sterile forceps, and incubated for 24 hours at 37°C. Staphylococci epidermidis and saprophyticus were separated using Novobiocin, Bacitracin, and Polymycin B discs. To differentiate Hominis from epidermidis staphylococci, Phosphomycin and Deferoxamine antibiotics were used, and staphylococci were decided to be epidermidis. The results obtained were examined according to the latest standards of Clinical and Laboratory Standards Institute (CLSI). Staphylococcus aureus strain ATCC 25923 was used as negative control in all tests.

Kirby-Bauer disc diffusion technique was used to determine sensitivity of isolates to penicillin 10 µg, cefoxitin 30 µg, oxacillin 1 µg, ceftriaxone 30 µg, cefotaxime, cefazolin 30 µg, and cephalexin 30 µg (All from Mast-England). In this technique, first, a suspension is prepared from 12 or 24-hour bacterial culture with opacity of 0.5 McFarland. This suspension is then cultured on a 5mm thick Mueller Hinton medium (Merck-Germany). Discs are placed onto the medium with sterile forceps, and incubated for 24 hours at 37°C. To minimize contamination, discs were placed on the plate at suitable intervals using Disc Dispenser device (Mast-England). After 24 hours incubation at 35 °C, diameter of non-growth zones was examined according to the latest standards of Clinical and Laboratory Standards Institute (CLSI).

Micro-tubes containing clinical BHI isolates preserved at 20 °C on Sabouraud Blood Agar medium were linearly cultured, and incubated for 24 hours at 37°C. Then, a number of colonies from each cultured isolate were inoculated into 5ml of Luria Bertani Broth culture medium (that had already been divided into as many cap glass tubes as isolates), and incubated for 20 hours at 37 °C. Next, tubes were removed from incubator after 20 hours. From the resulting culture medium, 1.5cc was poured into cap plastic microtubes, and genomic DNA extraction stages were performed using extraction kit (from Sina-gene Company).

Primers procured from Macro-gene Company, ordered by Pishgam Company-Iran were diluted to initial density (100 pico molar) by adding distilled water, and then direct and reverse primers were prepared at 15 pico-molar dilution. After refrigeration of primers at +4 °C for 4 hours, stock dilutions were kept at -20 °C for further tests. Extracted DNAs were used in PCR tests. The following primers were used in proliferation of blaZ gene in study samples.

Table 1: Specific primers used in identification of blaZ and mecA genes

Target genes	Primer	Sequence length	Size (base pair)	Reference
blaZ	blaZ F	TACAACTGTAATATCGGAGGG	310	Sidhu et al. (19)
	blaZ R	AGGAGAATAAGCAACTATATCATC		
mecA	mecA F	AAAATCGATGGTAAAGGTTGGC	533	Nahaei et al. (20)
	mecA R	AGTTCTGCAGTACCGGATTTGC		

For the PCR reaction, 25 µl of the final solution was used, which included 1 µl of sample DNA, 1 µl of each primer of 15 pico molar concentration, 12.5 µl of master mix of 2× and 1.5×mM MgC12 (Ampliqon Company-Germany) that contained Tris-Hcl PH8.5, (NH4) SO4, 3mM Mgcl2, MmdNTP 4.4, 0.2% Tween 20, 0.2 unit Ampliqon polymeras, Insert red dye and stabilizer. Deionized distilled water was used to make the final volume. PCR mixture with no DNA model was used as negative control (14). PCR test was performed for mecA gene using thermocycler (BioRad-America), which included initial denaturation at 95° C for 3 minutes, 35 denaturation cycles at 94° C for 45 seconds, primer binding stage at 55

°C for one minute, and proliferation of the target piece at 72 °C for one minute and 30 seconds. The final proliferation was performed at 72 °C for 7 minutes for blaZ gene and mecA, which involved initial denaturation at 95 °C for 3 minutes, 35 denaturation cycles at 94 °C for 30 seconds, and proliferation of the target piece at 72 °C for 30 seconds. The final proliferation was performed at 72 °C for 3 minutes. PCR products were separated from one another by electrophoresis using agarose gel 1.5%. To this end, 5µl of final PCR product was electrophoresed in agarose gel 1.5% in 0.5X buffer. Gel was dyed by adding and stirring 5µl of Gel Red solution (Biotium-America), and then observed in ultra-violet light at 260nm wavelength using trans-illuminator. Size of the products was determined using molecular fermentas marker (Thermofisher-America) with a sequence of 100 base pairs. Finally, the resulting gel was photographed with Gel Dock (CCD-Tab1- Kiagen-Iran).

Data obtained were analyzed in SPSS-16 using descriptive statistics including frequency, percentage, and mean. Chi-square test was used to compare qualitative results, independent t-test to compare quantitative results. Significance level was considered $P \leq 0.05$.

Results

The present study showed that the highest resistance in isolates obtained from saprophyticus and epidermidis staphylococci to beta-lactam antibiotics related to penicillin, with more than 90% resistance in both strains. Next to penicillin, oxacillin and cefoxitin were respectively among the most resistant to beta-lactam antibiotics in this strain (Table 1). Most isolates obtained from coagulase-negative related to urine culture (49.49%, and 55.69%), followed by isolates relating to urine catheters (20.25% and 36.36%) (Table 2). The molecular results indicated relatively high prevalence of studied genes in coagulase-negative isolates. In other words, frequency of mecA and blaZ genes was 29.11% and 59.49% respectively in staphylococcus saprophyticus, and 48.97% and 75.25% respectively in staphylococcus epidermidis (Table 3). This shows a significant increase in methicillin-resistant strains and blaZ carrying strains.

Table 2: Antibiotic-resistance pattern of saprophyticus and epidermidis staphylococci isolates

Antibiotic	Coagulase-negative staphylococci						P-value (P<0.05)
	Epidermidis staphylococcus			Saprophyticus staphylococcus			
	Resistant (%)	Semi-sensitive (%)	Sensitive (%)	Resistant (%)	Semi-sensitive (%)	Sensitive (%)	
Ceftriaxone	84 42.42	0	114 57.57	24 30.37	5 6.9	50 63.25	0.24
Cephalexin	43 21.71	1 0.57	154 77.77	18 22.74	23 29.11	38 48.10	0.19
Cefexime	55 27.77	0	143 72.22	20 25.31	5 6.38	54 68.35	0.33
Cefoxitin	86 43.43	0	111 56.06	22 27.84	0	57 72.26	0.0045
Penicillin	85 42.29	0	110 55.55	26 32.91	2 2.8	51 64.55	0.03
Oxacillin	84 42.42	0	112 56.56	23 29.11	0	56 70.88	0.045
Ciprofloxacin	61 30.81	9 45.23	128 16.45	13 16.45	21 26.58	45 56.96	0.11

Table 3: Frequency of mecA and blaZ genes in saprophyticus and epidermidis staphylococci isolates

Genes	Coagulase-negative staphylococci			
	Epidermidis staphylococcus (n=198)		Saprophyticus staphylococcus (n=79)	
	With gene (%)	Without gene (%)	With gene (%)	Without gene (%)
mecA	96	102	23	56
	48.97	51.51	29.11	70.88
blaZ	149	49	47	32
	75.25	24.74	59.49	40.50

Table 4: Frequency of saprophyticus and epidermidis staphylococci isolates according to sample type

Sample	Coagulase-negative staphylococci (n=277)	
	Epidermidis staphylococcus (n=198)	Saprophyticus staphylococcus (n=79)
Wound excretions	11	17
	6.14	21.51
Urine	98	44
	49.49	55.69
Swab	17	2
	8.58	2.53
Catheter tip	72	16
	36.36	20.25

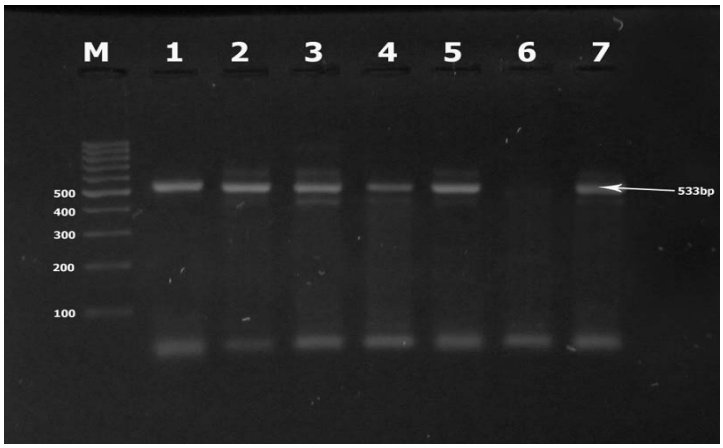

Figure 1: Electrophoresis gel result for mecA gene in clinical samples of coagulase-negative staphylococcus
Well M, molecular marker of 100 base pairs length
Wells 1 to 5 positive samples in terms of mecA gene, well 6 negative control, and well 7 positive control

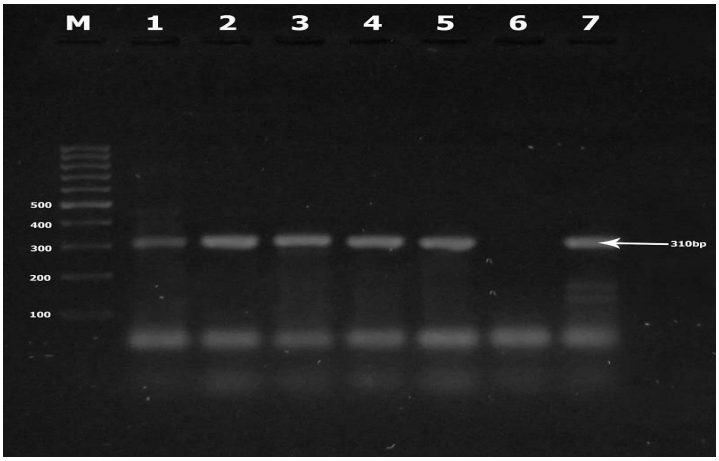

Figure 2: Electrophoresis gel result for blaZ gene in clinical samples of coagulase-negative staphylococcus
Well M, molecular marker of 100 base pairs length
Wells 1 to 5 positive samples in terms of blaZ gene, well 6 negative control, and well 7 positive control

Discussion

Considering the increasing trend in antibiotic-resistance of bacteria causing nosocomial infections, the high prevalence of resistance to beta-lactam in coagulase-negative group has turned into a worrying matter, such that, in terms of phenotyping 92% of staphylococcus epidermidis and 96% of staphylococcus saprophyticus show resistance to penicillin, which is comparable to the results obtained in a study conducted by Z Xu et al in England (14). Next to penicillin, the highest resistance was to oxacillin and cefoxitin in antibiotics studied, which is similar to Fessler et al. observations in Germany (21). The results obtained in a study conducted by Nahaei et al. in Iran were indicative of varying range of resistance to beta-lactams in saprophyticus and epidermidis staphylococci, which agrees with the present study results (17). In a study conducted in India by Makki et al. on coagulase-negative staphylococci, the least species detected were saprophyticus and epidermidis staphylococci, which disagrees with the present study (7). Among antibiotics studied, resistance to cefazolin was the lowest, which concurs with the results obtained in studies conducted by Kiato T et al. in Japan and Nahaei et al. in Iran (22). Bacterial isolates were mostly obtained from urine samples and least from wounds and wound excretions, which is similar to the results obtained by Raei et al. (13). In the present study, frequency of methicillin-resistance was 29.11% in saprophyticus and 48.97% in epidermidis staphylococci, which is similar to the results obtained in a study conducted by deMattos et al. in Brazil (23). In terms of frequency, blaZ carrying strains were observed in both epidermidis (75.25%) and saprophyticus (20.25%) staphylococci. A study conducted by Olsen et al. in Denmark on classification of a variety blaZ and their frequencies in coagulase-negative groups indicated a dramatic increase in this gene among various saprophyticus and epidermidis staphylococci strains, which concurs with the present study findings (15). Molecular results disagree with the results obtained from antibiogram phenotypic tests. This difference is mostly due to false sensitivity in methicillin-resistant strains (24). In most cases, antibiogram phenotypic disc diffusion technique is inappropriate for determining treatment protocol for these infections (25). Studies conducted in Iran on resistance of staphylococci to beta-lactam antibiotics have mostly relied on phenotypic techniques such as antibiogram test for determining minimum inhibitory concentration and beta-lactamase synthesis, and few studies have been specifically conducted on epidermidis and saprophyticus staphylococci. On the other hand, beta-lactamase synthesis poses a serious risk in the treatment of infections resulting from beta-lactamase producing organisms. Thus, rapid accurate molecular tests are considered imperative for choosing the right antibiotic and determining the right course for controlling these infections.

Conclusion

Given the prevalence of mecA and blaZ and the phenotypic test results in coagulase-negative staphylococci, the prevalence of resistant strains to various antibiotic groups is rising. Moreover, based on the conflicting results from phenotypic and genotypic methods in detecting beta-lactam resistant coagulase-negative staphylococci, it can be concluded that phenotypic methods alone are unable to definitively detect resistant strains. Besides, due to the high administration of beta-lactam antibiotics for the treatment of infections associated with negative-coagulase, a definitive and accurate detection should be reached, so as to hasten treatment, and also prevent inappropriate administration of antibiotics.

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Conflict of interests

The Authors declare that there is no conflict of interest in this paper.

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