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# Distribution of Aminoglycoside Resistance Genes in Coagulase-Negative Staphylococci Isolated From Hospitalized Patients

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## Abstract

**Background:** The incidence of coagulase-negative staphylococci (CNS) isolates, as causes of hospital-acquired infections, is increasing annually and it is a truly global challenge.

**Objectives:** The current study aimed at assessing aminoglycoside resistance genes among CNS isolated from hospitalized patients and investigating the prevalence of methicillin and aminoglycoside resistance genes using molecular methods.

**Methods:** A total of 103 species of coagulase negative staphylococci were isolated from clinical specimens from August 2013 to November 2014. All the specimens were identified using conventional microbiological methods including colony morphology, Gram staining, catalase, and slide and tube coagulase. The isolates were subjected to the API-20 Staph identification kit. Antibiotic susceptibility tests were performed using Kirby-Bauer disc diffusion method. The methicillin and aminoglycoside resistant genes among coagulase negative staphylococci were detected by polymerase chain reaction (PCR) and sequencing methods.

**Results:** The most frequent clinically isolated coagulase negative staphylococci species were *Staphylococcus hominis* and *S. haemolyticus* (51.9%), *S. epidermidis* (21.7%), *S. lugdunensis* (9.8%), *S. intermedius* (4.9%), *S. saprophyticus* and *S. simulans* (3.9%), *S. warneri* (2.9%), as well as *S. chromogenes*, *S. sciuri*, *S. caprae*, *S. schleiferi*, and *S. xylosus* (0.98%). The overall rate of methicillin resistance among CNS species in the present study was 89 (86.4%). The resistance of CNS isolates against tested antibiotics was as follows: 74 (71.8%) to cefoxitin, 54 (52.4%) to kanamycin, 51 (49.5%) to gentamicin, 45 (43.7%) to tobramycin, and 30 (29.1%) to amikacin. The prevalence of aminoglycoside resistance genes such as *ant* (4')-Ia, *aac* (6')/*aph* (2") and *aph* (3')-IIIa were 89 (86.4%), 87(84.5%), and 68(66%), respectively.

**Conclusions:** The presence of antibiotic resistance among coagulase negative *Staphylococcus* species, which cause nosocomial infections, has increased. Therefore, identification of coagulase negative staphylococci within 24 hours after development of enzymatic tests would be very useful in any clinical microbiology laboratory for effective treatment. Resistance to antibiotics, including aminoglycosides, develops quickly in CNS species where these antimicrobial agents are widely used. Thus, a better understanding of antibiotic resistance of CNS species is essential to eliminate antibiotic resistance in hospitals and society.

*Keywords:* Coagulase Negative Staphylococcus, API Test, Aminoglycoside Resistance Genes

## 1. Background

Coagulase-negative staphylococci (CNS) infections are commonly associated with medical-device related infections, such as heart valves, pacemaker lines and boxes, cerebrospinal shunts, prosthetic joints, and also in immune-compromised patients, such as the patients with cancer and the ones undergoing chemotherapy and dialysis (1, 2).

Meanwhile, aminoglycoside antibiotics are one of the most used antibiotics in clinical practice that play an important role in the treatment of patients. Resistance to aminoglycosides develops quickly in CNS species among different clinical settings (3).

Inactivating the drug could be caused by aminoglycoside-modifying enzymes (AMEs) encoded within mobile genetic elements (4). Modifying enzymes are encoded with several distinct genes including aminoglycoside-6'-N-acetyltransferase/2"-O-phosphoryltransferase [*aac*(6')/*aph*(2")], aminoglycoside-4'-O-nucleotidyltransferase I [*ant*(4')-I], and aminoglycoside-3'-O-phosphoryltransferase III [*aph*(3')-III] (5, 6). Subclasses are defined by the position of the catalytic sites, at a hydroxyl group (*aph* and *ant*) or at an -NH2 group (*aac*) (7). The *ant*(4')-I mediates resistance to neomycin, kanamycin, tobramycin, and amikacin in staphylococci; while, *aph*(3')-III can mediate resistance to neomycin and kanamycin (7). The distribution of

AME genes largely depends on bacterial species, but the combination of AMEs is correlated with the use of aminoglycosides in specific geographic regions (8).

## 2. Objectives

The current study aimed at describing the identification of isolated CNS and investigating the presence of genes encoding AMEs to the aminoglycoside susceptibility pattern and methicillin resistance.

## 3. Methods

### 3.1. Bacterial Strains

From August 2013 to November 2014, a total of 103 CNS isolates were collected from clinical specimens of the patients admitted to 6 major hospitals located in different regions of Tehran, Iran. The isolates were identified by their phenotypic characteristics such as colony morphology, Gram staining, catalase, and slide and tube coagulase test results.

### 3.2. Identification and Susceptibility Testing

The identification of CNS species was carried out using API-Staph system. Each isolate was grown overnight on Muller-Hinton agar (Merck, Darmstadt, Germany) and added in an aliquot of the API-Staph medium to each tube of the gallery, enclosed within rigid plastic tray (API bioMerieux, France). The galleries were incubated for 24 hours at 37°C. The tested biochemical reactions included the fermentations of glucose, fructose, mannose, maltose, lactose, trehalose, mannitol, xylitol, melibiose, raffinose, xylose, sucrose, a-methyl glucoside, and N-acetyl glucosamine, the reduction of nitrate, the hydrolysis of arginine and urea, phosphatase activity, and the formation of acetoin from pyruvate. At the end of the incubation period, to demonstrate alkaline phosphatase activity, acetylmethyl carbinol formation, and nitrate reduction, active reagents, containing $\beta$-naphthyl phosphate, sodium pyruvate, and potassium nitrate were added to the cupules.

### 3.3. Antibiotic Susceptibility Testing

All the isolates were tested, using a disc diffusion method, to evaluate resistance to gentamicin (GEN: 10 $\mu$g), tobramycin (TOB: 10 $\mu$g), kanamycin (KAN: 30 $\mu$g), amikacin (Ami: 30 $\mu$g), and cefoxitin (CEF: 30 $\mu$g) (MAST, Merseyside. U.K)according to the clinical and laboratory standards institute (CLSI) guidelines (9).

### 3.4. DNA Extraction and Amplification

DNA was extracted by a kit (Qiagen, Hilden, Germany). The polymerase chain reaction (PCR) was carried out in Intellectica thermo cycler (Techne, UK) by the PCR Master Kit (Cinna Clone Inc., Iran), according to the manufacturer's guidelines. Primer sequences were used to detect *mecA*, *ant* (4´)-*Ia, aac* (6´)-*Ie*/*aph* (2˝), and *aph* (3´)-*IIIa* genes; the fragment sizes are presented in Table 1. PCR condition was as follows: Initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, annealing for *mecA* gene 1 minute at 57°C, *aac* (6')/*aph* (2") 45 second at 56°C, *ant* (4')-I 30 second at 48°C, and *aph* (3')-III 45 second at 57.5°C, extension at 72°C for 30 second. *Staphylococcus aureus* USS1504, S. aureus BM3002/ piP52, and *Enterococcus faecalis* BM6217 were used as the positive controls for *mecA*, *ant* (4′)-*Ia, aph* (3′)-*IIIa, aac* (6′)/*aph* (2′′), respectively.

The final extension step was continued for another *mecA* gene for 8 minutes at 72°C and the rest of aminoglycoside gene for 60 seconds at 72°C. DNA fragments were analyzed using electrophoresis in a 1% agarose gel at 95 V for 45 minutes in 1 X Tris-Borate-ethylenediaminetetraacetic acid (TBE) containing ethidium bromide. Finally, sequencing of forward strand was performed by the Bioneer Company (Korea). The nucleotide sequences were analyzed using Chromas 1.45 and MEGA-4 software and BLAST at NCBI.

### 3.5. Statistical Analysis

Statistical analysis was performed using SPSS for Windows (version 17.0) (SPSS Inc., Chicago, IL) running Chi-squared test.

## 4. Results

A total of 103 strains of CNS (blood = 27, wound = 37, pus = 1, urine = 15, central nervous system (CNS) = 2, catheter = 4, sputum = 9, bronchoalveolar lavage fluid = 2, prosthetic joints = 1, and body fluids = 5) were recovered from 6 hospitals in Tehran, Iran.

From a total of 103 strains, 102 were identified using the API test; only one isolate, obtained from a prosthetic joint, could not be identified. Therefore, 102 strains were analyzed. Table 2 presents the identification list of the most likely species among hospitalized patients in Iran.

In vitro susceptibility of the CNS isolates were tested to 5 antibiotics. A total of 54 isolates (52.4%) were resistant to kanamycin. Some strains of CNS were detected with intermediate resistance to kanamycin 4 (3.9%), tobramycin, and amikacin 2 (1.9%) (Table 3).

According to the results of the present study, the rate of resistance to kanamycin, gentamicin, tobramycin, and amikacin were 52.4%, 49.5%, 43.7% and 29.1%, respectively.

**Table 1.** PCR Primers Used to Detect Aminoglycoside Resistance Genes

| Primer (5' 3') | Amplicon Size, bp | Gene | References |
|---|---|---|---|
| GTA GAA ATG ACT GAA CGT CCG ATA A | 310 | *mecA* | (7) |
| CCA ATT CCA CAT TGT TTC GGT CTA A | | | |
| AATCGGTAGAAGCCCAA | 135 | *ant*(4´)-Ia | (7) |
| GCACCTGCCATTGCTA | | | |
| CCAAGAGCAATAAGGGCATACC | 222 | *aac*(6´)-*Ie*/*aph*(2˝) | (7) |
| CACACTATCATAACCACT | | | |
| CTTGATCGAAAAATACCGCTGC | 269 | *aph*(3´)-*IIIa* | (7) |
| TCATACTCTTCCGAGCAAA | | | |

**Table 2.** Frequency of CNS Species Identified by Commercial Kits

| Organisms | No. of Isolates (%) | No. of Isolates From | | | |
|---|---|---|---|---|---|
| | | Wound Infection | Endocarditis and Blood Cultures | Urinary Tract Infections | Miscellaneous Infections |
| ***Staphylococcus hominis* and *Staphylococcus haemolyticus*** | 53 (51.9) | 21 | 5 | 11 | 16 |
| ***Staphylococcus epidermidis*** | 22 (21.7) | 5 | 15 | 1 | 1 |
| ***Staphylococcus lugdunensis*** | 10 (9.8) | 5 | 2 | 2 | 1 |
| ***Staphylococcus intermedius*** | 5 (4.9) | 2 | 2 | | 1 |
| ***Staphylococcus saprophyticus* and *Staphylococcus simulans*** | 4 (3.9) | 1 | 2 | 0 | 1 |
| ***Staphylococcus warneri*** | 3 (2.9) | 1 | 1 | 0 | 1 |
| ***Staphylococcus chromogenes*** | 1 (0.98) | 0 | 0 | 1 | 0 |
| ***Staphylococcus sciuri*** | 1 (0.98) | 0 | 0 | 0 | 1 |
| ***Staphylococcus caprae*** | 1 (0.98) | 1 | 0 | 0 | 0 |
| ***Staphylococcus schleiferi*** | 1 (0.98) | 1 | 0 | 0 | 0 |
| ***Staphylococcus xylosus*** | 1 (0.98) | 0 | 0 | 0 | 1 |
| **Total identified no. (%)** | 102 (100.0) | 37 | 27 | 15 | 23 |

Also, with regard to Table 4, the proportion of methicillin-resistant CNS (MRCNS), which showed resistance to the aminoglycosides in the present study, was higher than that of the methicillin-sensitive CNS (MSCNS) isolates (Figure 1).

The highest prevalence of aminoglycoside resistance genes among CNS isolates was *ant* (4')-I occurring in 87.6% of MRCNS species (Figure 2). However, the rate of *aac* (6')/*aph* (2") was very high (Figure 3). The least common gene was *aph* (3')-III, found in 66.3% of MRCNS species (Figure 4). The rate of coexistence of *aac* (6')-*Ie-aph* (2") with *aph* (3')-*IIIa*, and *aac* (6')-*Ie-aph* (2") with *ant* (4')-*Ia* was 65 (63%) and 77 (74%), respectively. The rate of coexistence of 3 AME genes was 57 (55.3%).

## 5. Discussion

Antibiotic resistance remains a major threat to public health worldwide (10). Although other studies showed that the most prevalent spices was *S. epidermidis* (11, 12), the results of the present study showed that *S. hominis* and *S. haemolyticus*, as the most important causes of nosocomial infections, had the highest prevalence (10). Insufficient

**Table 3.** Antimicrobial Susceptibilities of 103 CNS Isolates to 5 Antibiotics[a]

| Hospital | Antibiotics | | | | |
|---|---|---|---|---|---|
| | Tobramycin | Kanamycin | Amikacin | Gentamicin | Cefoxitin |
| K | 17 (65.4) | 20 (76.9) | 20 (76.9) | 20 (76.9) | 25 (96.2) |
| M | 7 (30.4) | 7 (30.4) | 1 (4.3) | 8 (34.8) | 12 (52.2) |
| P | 8 (33.3) | 9 (37.5) | 4 (16.7) | 8 (33.3) | 18 (75) |
| MO | 6 (46.2) | 8 (61.5) | 2 (15.4) | 7 (53.8) | 9 (69.2) |
| S | 1 (11.1) | 3 (33.3) | 0 | 2 (22.2) | 3 (33.3) |
| T | 6 (75) | 7 (87.5) | 3 (37.5) | 6 (75) | 7 (87.5) |
| Total | 43.7 | 52.4 | 29.1 | 49.5 | 71.8 |

Abbreviations: K, Khatam, M; Motahari; MO, Mofid; P, Pars; S, Sasan; T, Taleghani.
[a]Values are expressed as No (%).

**Table 4.** The Distribution of Aminoglycoside Resistance Genes in Isolates of CNS in Relation to Methicillin Resistance

| Resistant Gene | Methicillin Sensitive CNS (n = 14) | Methicillin Resistance CNS (n = 89) |
|---|---|---|
| ***aac*(6')/*aph*(2")** | 13 (92.9) | 74 (83.1) |
| ***aph*(3')-III** | 9 (64.3) | 59 (66.3) |
| ***ant*(4')-I** | 11 (78.9) | 78 (87.6) |

Abbreviations: MRCNS: methicillin-resistant CNS; MSCNS, methicillin susceptible CNS.

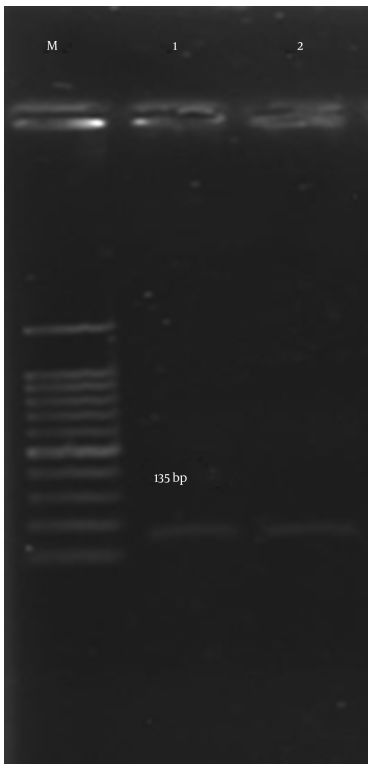
**Figure 1.** Detection of Genes Encoding *mecA* Gene in CNS Isolates by PCR


M, a 100 bp DNA ladder (Fermentas); lane 1, positive control; lane 2, negative control; lane 3 patient sample.

hand hygiene and inadequate disinfection and/or sterilization of medical instruments and surfaces may also be assumed as causes of the distribution of coagulase-negative Staphylococci (CoNS) such as *S. hominis* and *S. haemolyticus* in the hospital settings (13). As previously mentioned, excessive consumption of different antibiotics has led to the emergence of multi-drug resistance in the developing countries (14). Aminoglycosides are an important group of antibiotics in the treatment of serious bacterial infections, especially Gram negative bacteria, but the current reports indicated the emergence of resistance to aminoglycosides in CNS isolates in different parts of the world (15).

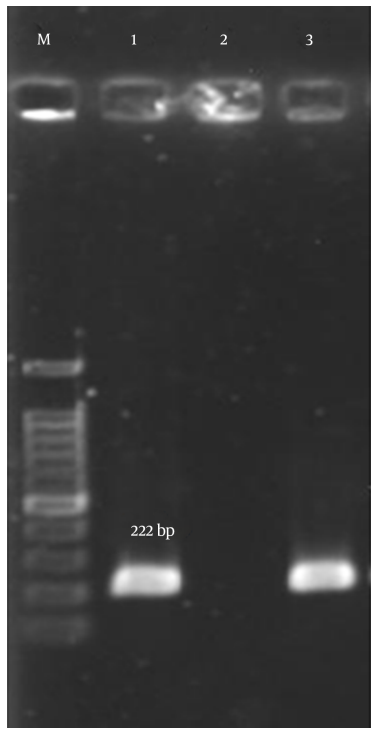
The methicillin resistance rate of 86% (89/103) among the study CNS isolates was higher than that of reported in Colombia (73%) (16), Egypt (75%) (17), and Brazil (77%) (18), which could be due to the overuse of antibiotics that eradicate MSCNS and facilitate MRCNS colonization. Furthermore, activity in screening, outbreak investigations, and contact tracing regarding methicillin-resistant coagulase-negative Staphylococci (MRCoNS) may differ between Iran and the abovementioned countries. Despite many previous studies, which had shown the more common rate of *aac* (6')/*aph* (2") gene (19-21), in the current study, the highest prevalence of aminoglycoside resistance genes was related to *ant* (4')-I (87.6%). This finding was similar to the ones reported from Kuwait (88%) (21) and Japan (84.5%) (22). The 2nd most prevalent AMEs gene was *aac* (6')-*Ie-aph* (2"), which can inactivate gentamicin, kanamycin, tobramycin, neomycin, and amikacin (23). The 3rd gene was *aph* (3)-*IIIa*,

**Figure 2.** Detection of Genes Encoding *ant* (4')-I Gene in CNS Isolated by PCR



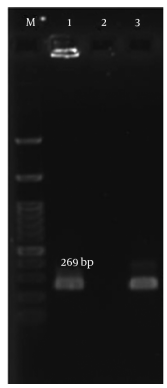

M, a 100 bp DNA ladder (Fermentas); lane 1, positive control; lane 2, patient sample.

**Figure 3.** Detection of Genes Encoding *aac*(6')-*aph*(2") Gene in CNS Isolates by PCR




M, a 100 bp DNA ladder (Fermentas); lane 1, positive control; lane 2, negative control; lane 3, patient sample.

**Figure 4.** Detection of Genes Encoding *aph*(3')-III in CNS Isolates by PCR




M, a 100 bp DNA ladder (Fermentas); lane 1, positive control; lane 2, negative control; lane 3, patient sample.

which inactivates kanamycin and amikacin (23). Although it was found to be the lowest in the current study (66.3%), its amount was higher than reported by other studies such as those of Ghotaslou (19.3%) (15) and Schmitz (14%) (24). Differences between reports from different countries could be due to differences among the isolates and geographical regions. The probable coexistence of all 3 enzymes was detected in the current study isolates, similar to the other 2 researches. They showed that most of the isolates carried the genes for *aac* (6')-*aph* (2"), *ant* (4'), and *aph* (3'), and only a few of them carried genes for single enzymes due to the presence of the same plasmid or transposon encoding the AMEs in CNS species (21). According to the existence of 2 *mecA* and AMEs genes, many studies showed a correlation between aminoglycoside and methicillin resistance (25, 26).

In general, identification of the species and their resistance patterns contribute to appropriate prescription of antibiotics and accordingly, decrease of antibiotic resistance rate and prevention of widespread resistant genes among CNSs. Because the resistance of CNS to multiple antimicrobial agents varies from one species to another, determining susceptibility to antibiotics for each species allows the researchers to intercept outbreaks of hospital infections caused by CNS. Meanwhile, prescription of antibiotics, including aminoglycoside, should be done with more surveillance. Thus, it seems likely that phenotypic

measures, rapid and molecular methods, as well as detection of the susceptibility of bacterial isolates to antimicrobial agents contribute to appropriate treatment of the patients.

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## Footnotes

**Authors' Contribution:** Mehdi Goudarzi and Hossein Goudarzi: study concept and design, development of the study, data interpretation, and manuscript revision; Mehdi Goudarzi and Fattaneh Sabzehali: Phenotypic and molecular studies, and manuscript drafting; Fattaneh Sabzehali: performing experimental procedures; Hadi Azimi: participated in the acquisition of data, statistical analysis, and language revision of the manuscript; Mehdi Goudarzi and Hossein Goudarzi: Study supervision; All authors read and approved the final manuscript.

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