Identification of hemolysin encoding genes and their association with antimicrobial resistance pattern among clinical isolates of coagulase-negative Staphylococci

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Abstract

Objective: Coagulase-negative staphylococci (CoNS) are considered opportunistic pathogens which capable of producing several toxins, enzymes and resistance genes. The current study aimed to determine the occurrence of different hemolysins genes and patterns of antibiotic resistance among CoNS species.

Results: The highest frequency of antibiotic resistance was observed against cefoxitin in 49 isolates (53.8%), and the lowest resistance was against novobiocin in 5 isolates (5.5%). None of the isolates was resistant to vancomycin. The prevalence of hla, hla_yidD, hld, and hlb genes was: 87.9%, 62.6%, 56%, and 47.3%, respectively. The hla/yidD and hld genes were detected in 69.4% of S. epidermidis and the hla gene in 94.6% of S. haemolyticus isolates; the hlb gene was detected in 53.1% of the S. epidermidis isolates. The mecA gene was identified in 50 (55%) of the CoNS isolates. In conclusion, the results of statistical analysis showed that the hld gene had a significant association with resistance to levofloxacin and erythromycin antibiotics, the hlb with clindamycin resistance and the hla/yidD with rifampicin and novobiocin resistance. The results of this study showed that there is a significant relationship between hemolysin encoding genes and antibiotic resistance patterns; therefore, detection of virulence factors associated with antibiotic resistance has become a significant issue of concern.

Keywords: CoNS, Hemolysin, Antibiotic resistance

Introduction

Coagulase-negative staphylococci (CoNS) are considered as opportunistic pathogens that cause a variety of infections, particularly among immunocompromised, long-term hospitalized patients, preterm infants and in patients with indwelling or different implant polymer bodies [1–3]. Among various CoNS species, Staphylococcus epidermidis, Staphylococcus haemolyticus, and Staphylococcus saprophyticus have been confirmed to be responsible agents for the vast majority of nosocomial infections [4]. Treatment of CoNS infections has become more complicated, as many isolates in hospitals show high rates of resistance to multiple antimicrobial agents of clinical relevance [5]. CoNS is also a reservoir for resistance genes that can be transmitted to other pathogens [6]. About 80–90% of CoNS isolates associated with nosocomial infections are methicillin-resistant coagulase-negative Staphylococci [7]. Most antibiotic resistance genes are carried on a plasmid and more often found in methicillin-resistant [8]. CoNS are capable of producing several toxins and enzymes characteristically associated with Staphylococcus aureus such as hemolysins, which
are responsible for the invasion of host cells [9, 10]. Hemolysins of *staphylococci* are categorized into four different types, including alpha (α), beta (β), gamma (γ), and delta (δ). The α-toxin is encoded by the *hla* and acts as a pore-forming cytotoxin (PFT) which actsives against a wide array of human cells. Pathogenicity of this toxin is due to hemolytic, dermonecrotic, and neurotoxic effects [11–14]. β-toxin which encoded by the *hld* gene is known as Mg2+—dependent sphingomyelinase. Incubation at temperatures below 10 °C intensifies the cytolytic activity of β-toxin; thus, it is often referred to as the ‘hot–cold’ hemolysin [15, 16]. The *hld* gene encodes a 26 amino acid peptide, which is referred to as delta (δ) hemolysin. This toxin with its detergent function degrades erythrocytes. The delta toxin may cause intestinal diseases that can vary from acute diarrhea to severe enteritis [15, 17, 18]. Because the association between antimicrobial resistance and virulence factors of CoNS isolates had never been done before as well as in a similar work in *staphylococcus aureus* strains observed a significant relationship between antibiotic resistance and hemolysin genes thus we made attempt to determine this association in this study.

**Main text**

**Methods**

**Identification of CoNS isolates**

A total of the 91 CoNS isolates were collected from various clinical specimens submitted to three teaching hospitals (including Beheshti, Besat, and Farshchian) located in Hamedan, Iran, from September 2017 to November 2018. All isolates were investigated by conventional methods. The origins of the isolates were as follows: blood, urine, catheters, and wounds. This study was approved by the Ethics Committee of Hamadan University of Medical Sciences (Code No: IR.UMSHA.REC.1396.827).

**Antibiotic susceptibility testing**

The Antibiotic susceptibility testing of 91 CoNS species carried out using the standard disk agar diffusion (DAD) method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. Mueller–Hinton agar culture medium (Merck, Germany) was inoculated with a saline suspension of isolated CoNS equivalent to McFarland 0.5 standard. After that, antibiotic discs were placed on the surface of the agar. After 16-18 h incubation at 37 °C, CoNS isolates were categorized to be resistant, moderately resistant, or sensitive based on the size of growth inhibition zone. The antimicrobial agents used in current study were as follows: for the following antimicrobial agents: chloramphenicol (30 µg), cefoxitin (30 µg), clindamycin (2 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), levofloxacin (5 µg), novobiocin (5 µg), rifampicin (5 µg), trimethoprim-sulfamethoxazole (25 µg) and vancomycin (30 µg) (MAST, Merseyside, UK). *S. aureus* ATCC33591 was used as a quality control.

**DNA extraction from isolates**

CoNS Chromosomal DNA was extracted by boiling method. Quality of extracted DNA was assessed by the Nanodrop ND–1000 (Nanodrop Technologies, Inc., Wilmington, DE, USA).

**Detection of hemolysin genes**

Identify of hemolysin encoding genes was performed by Multiplex PCR (*hla haem, hla/yidD epid* and *hld_epli*) and single PCR (*hld_epi*) using specific primers (Additional file 1: Table S1). The PCR conditions included an initial denaturation at 94 °C for 4 min, followed by amplification; 30 cycles at 94 °C for 1 min, 58 °C for 1 min (45 °C-1 min for *hld_epi*), 72 °C for 1 min and 72 °C for 5 min. *hla_haem: S. haemolyticus* ATCC29970, *hld_epli: S. epidermidis* ATCC 12228 and *hla/yidD_epid: S. epidermidis* ATCC 12228, were used as control.

**Detection of mecA gene**

PCR assay was designed to amplify the *mecA* gene, using specific primers which are presented in Table 1. Amplification involved an initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 60 s), with a final extension step (72 °C for 7 min). *S. aureus* ATCC29247 was used as control.

**Statistical analysis**

Cramer’s V, Phi and Chi Square test were performed to assess of variables correlation. Phi and Cramer’s V have ranges from 0 to 1, where 1 indicates a significant association and 0 indicates no relationship. Interpretation of the Phi and Cramer’s V results: > 0; No or very weak, > 0.05 weak; > 0.10 moderate; > 0.15 strong; > 0.25 very strong. The Chi Square test was done by SPSS software version 20. P value < 0.05 was considered as statistically significant.

**Results**

**Isolation and prevalence of CoNS isolates**

Of the 91 clinical isolates of CoNS, 49 (53.8), 37 (40.7%) and 5 (5.5%) isolates were recognized as *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus*, respectively. Isolates were recovered from blood 39 (42.9%), urine 33 (36.3%), catheter 13 (14.3%) and wound 6 (6.6%).
Antimicrobial susceptibility testing
The results of the antibiotic susceptibility testing of the 91 CoNS isolates, including 50 isolates (55%) of the Methicillin-resistant CoNS (MR-CoNS) and 41 isolates (45%) of the Methicillin-susceptible CoNS (MS-CoNS) are presented in Table 1. Multi-drug resistance was identified among 96% (48 isolates) of the MR-CoNS isolates and 63.4% (26 isolates) of the MS-CoNS isolates. Among MR-CoNS strains, the most frequent resistant was to cefoxitin (98%). Among CoNS species, the highest resistance was observed for cefoxitin (53.8%). None of the CoNS species was identified as being resistant to vancomycin.

Prevalence of hemolysin genes among CoNS isolates
The distribution of hemolysin genes among CoNS isolates is presented in Table 2. Our results showed a high frequency of hemolytic activity among CoNS strains isolated from blood, followed by urine, catheter, and wound, respectively. 80 isolates (87.9%) with hla gene, 57 isolates (62.6%) with hla/yidD epid, 51 isolates (56%) with hld and 43 isolates (47.3%) with hlb. Only 15 isolates (16%) had one type of the hemolysin, included; 12, 1 and two hla, hlb and hla/yidD positive CoNS isolates, respectively. Two types of the hemolysins were identified in 32 (35%) of the strains, the greatest coexistence of genes was observed for the hla + hla/yidD gene combination (14%), predominating followed by; hla + hld = 12 (13%), hla + hlb = 4 (0.04%), hld + hla/yidD = 2 (0.02%) and hlb + hla/yidD = 1 (0.01%). 14 (15%) of the strains were carrying three types of the hemolysins; hla + hlb + hla/yidD, hlb + hld + hla/yidD = 4 (0.04%) and hla + hld + hla/yidD = 3 (0.03%). All hemolysin genes were detected in 23 (25%) of the identified isolates.

Prevalence of mecA gene among CoNS isolates
The distribution of mecA gene among CoNS isolates and the prevalence of hemolysin genes among MR-CoNS species are presented in Additional file 1: Table S2. S. epidermidis strains had the highest frequency of mecA gene. Among the MR-CoNS isolates, the hla gene was the most common with frequency of 90%, followed by the hla/yidD (60%), hld (58%) and hlb genes (48%).

| Antimicrobial agent | MR-CoNS (n = 50) | MS-CoNS (n = 41) |
|---------------------|-----------------|-----------------|
|                     | S. epidermidis (n = 26) | S. haemolyticus (n = 22) | S. saprophyticus (n = 2) | S. epidermidis (n = 23) | S. haemolyticus (n = 15) | S. saprophyticus (n = 3) |
| Cefoxitin           | 25              | 22              | 2               | 0               | 0               | 0               |
| Trimethoprim-sulfamethoxazole | 15          | 10              | 1               | 10              | 5               | 1               |
| Erythromycin        | 4               | 8               | 0               | 9               | 4               | 1               |
| Clindamycin         | 4               | 6               | 0               | 6               | 3               | 0               |
| Chloramphenicol     | 8               | 7               | 0               | 6               | 2               | 0               |
| Rifampin            | 0               | 0               | 1               | 1               | 1               | 0               |
| Levofloxacin        | 1               | 3               | 0               | 4               | 1               | 2               |
| Vancomycin          | 0               | 0               | 0               | 0               | 0               | 0               |
| Gentamicin          | 10              | 6               | 0               | 2               | 2               | 0               |
| Doxycycline         | 7               | 2               | 0               | 7               | 5               | 1               |
| Novobiocin          | 0               | 0               | 2               | 0               | 0               | 3               |

Table 1 Prevalence of antibiotic-resistant strains among MR-CoNS and MS-CoNS

Table 2 Prevalence of hemolysin encoding genes among various CoNS species and various clinical samples

| Source       | CoNS (n = 91) |
|--------------|---------------|
|              | S. epidermidis n = 49 | S. haemolyticus n = 37 | S. saprophyticus n = 5 | Total n (%) |
| Catheter     | 40 (81.6)     | 35 (94.6)      | 5 (100)            | 80 (87.9)   |
| Wound        | 34 (69.4)     | 23 (62.2)      | 0                  | 57 (62.6)   |
| Urine        | 26 (53.1)     | 17 (46)        | 0                  | 43 (57.3)   |
| Blood        | 34 (69.4)     | 14 (37.8)      | 3 (60)             | 51 (56)     |
Statistical analysis

Statistical analysis results are presented in Table 3. Statistically, a significant association between CoNS species and the occurrence of hld and hla/yiD genes was observed in this study. It was found a meaningful relationship between antibiotic resistance and the presence of the genes for alpha, delta and beta hemolysins.

Discussion

In the present study, S. epidermidis (53.8%) was the most clinically significant of the CoNS species and S. haemolyticus (40.7%) was identified as the second, which is in agreement with other reports [20–22]. The rate of the CoNS isolation was highest in blood (43%) followed by urine (36.3%), catheter (14.3%) and wound samples (6.6%). Aher et al. and Parashar et al. were also reported that the vast majority of CoNS species isolated from blood compared to urine [23, 24]. In our study, the highest resistance rate of the CoNS isolates was against methicillin (55%), followed by cefoxitin (53.8%). According to previous studies, which have found significant discrepancies in resistance ratio against cefoxitin with frequencies of 58% to 84.7% and high resistance to erythromycin (76.9%) and Trimethoprim-sulfamethoxazole (74.9%) [7, 25–27]. In current study, the resistance ratio to methicillin was determined in 50 (55%) of CoNS isolates; the findings were comparable to other studies [28–31], those reported resistance to methicillin with frequencies of 43.8%, 59.64%, 62.8%, and 73.3%, respectively. In this study, the prevalence rate of multiple antibiotic resistance was significantly higher among MR-CoNS isolates compared to MS-CoNS isolates which is similar to the results of Koksal et al. [31]. The results of our studies indicated that the mecA gene was identified in all CoNS isolates revealing phenotypical resistance to cefoxitin, and only one strain of mecA-positive S. epidermidis was phenotypically cefoxitin-susceptible, which is in agreement with the others [27, 32]. Like other studies [25, 33], this study show that all CoNS isolates were also completely sensitive to vancomycin. However, there are reports of the occurrence of decreased susceptibility to vancomycin in CoNS isolates [34, 35].

Out of the 91 CoNS isolates, the percentage of strains with hemolytic activity were the highest among S. epidermidis (53.8%), followed by S. haemolyticus (39.5%) which is in agreement with the findings of Cunha et al. and Pinheiro et al., [36, 37], but, in contrary with the findings of Akinkunmi et al., [20]. Among CoNS species, 94.6% and 81.6% of the S. haemolyticus and S. epidermidis strains were determined to be positive to the hla gene, respectively. The hlb gene was detected in 53% and 46% of the S. epidermidis and S. haemolyticus strains, respectively. On the other hand, according to Okee MS et al., the occurrence of hlb gene not observed in any of the CoNS species isolated and the hla gene found in only 20% of S. epidermidis, which is also in contrary to a similar survey carried out by Pinheiro et al, who demonstrated the emergence of the hla and hlb genes in 92.9% of the S. epidermidis strains and hla with the same frequency (91.7%) in S. hemolyticus strains [37, 38]. In this study, the largest number of the strains carrying the hld gene belonged to S. epidermidis with frequency of 69.4%, then S. hemolyticus (37.8%), according to Pinheiro et al, 95.3% of the S. epidermidis and none of the S. haemolyticus were carrying hld gene, [37]. In the comparative statistical analysis among the MR-CoNS and MS-CoNS isolates found no significant association between the emergence of the hemolysins and methicillin resistance (p value from 0.472 to 0.962). All the isolated MR-CoNS demonstrated hemolytic activity either alone or in combined forms, while only one of the MS-CoNS strains was nonhemolytic which belonged to a S. haemolyticus recovered from urine sample. Among MR-CoNS, the highest number of strains carrying hemolysin genes are isolated from the blood rather than urine. But in a similar study done by Motamed et al, S. aureus hemolytic isolates were identified more in blood and ulcers compared to urine and catheter samples [39].

According with the pattern of antimicrobial

Table 3 Statistical analysis results for determining possible relationship between the following variables and the presence of types of hemolysins genes

| Types of hemolysins | mecA gene | Clinical sample | CoNS species | Cli | Ery | Levo | Novo | Rif |
|---------------------|-----------|-----------------|--------------|-----|-----|------|------|-----|
| hla                 | P = 0.472 | P = 0.122       | P = 0.131    | –   | –   | –    | –    | –   |
| hla_yiD             | P = 0.627 | P = 0.545       | P = 0.009    | –   | –   | –    | P = 0.003 | Φ = 0.31 |
|                     |           |                 |              |     |     |      | P = 0.02 | Φ = 0.19 |
| hlb                 | P = 0.962 | P = 0.807       | P = 0.075    | P = 0.009 | Φ = 0.28 | – | – | – |
| hld                 | P = 0.678 | P = 0.954       | P = 0.014    | –   | P = 0.04 | Φ = 0.26 | P = 0.04 | Φ = 0.21 |

Statistically significant P values are in italic (P < 0.05)

CoNS coagulase-negative staphylococci, Cli clindamycin, Ery erythromycin, Rif rifampicin, Levo levofloxacin, Novo novobiocin
resistance in CoNS isolates found a significant association between the occurrence of \textit{hld} gene and resistance to levofloxacin and erythromycin (P = 0.04, $\phi = 0.21$ and P = 0.04, $\phi = 0.26$, respectively), the \textit{hla/yidD} and resistance to rifampicin and novobiocin (P = 0.02, $\phi = 0.19$ and P = 0.003, $\phi = 0.31$, respectively), as well as between \textit{hla} and clindamycin resistance (P = 0.009, $\phi = 0.28$). Our results showed that the frequency of hemolytic agent genes of \textit{hla}, \textit{hlb}, \textit{hld} were higher in cefoxitin resistant isolates compared to susceptible ones. The analysis of our results demonstrated that there is no significant association between different clinical samples and hemolysins which is similar to the results of studies conducted by Corredor Arias et al., in \textit{S. aureus} strains [40]. Arabestani et al., Lee et al., and Osman et al. have been proved significant associations between pathogenicity factors and antibiotic resistance [41–43].

**Conclusion**

The results of this study demonstrated the significant role of antibiotic resistance in different infections due to increasing resistance to methicillin and the other antimicrobial agents among CoNS isolates. This study showed that the \textit{hla} and \textit{hla/yidD} genes and resistance to methicillin, cefoxitin, and trimethoprim-sulfamethoxazole antibiotics are widely distributed among the majority of CoNS isolated from human.

**Limitations**

The number of bacteria isolates was rather low. If the number of bacteria was more, the results would be better and also we have to design another study which focus on gene expression of the resistance and virulence factors.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s13104-020-4938-0.

The Additional files included Additional file 1: Tables S1 and S2.

**Abbreviations**

CoNS: Coagulase-negative staphylococci; PFT: Pore-forming cytotoxin; BHI: Brain Heart Infusion; DAD: Disk agar diffusion; CLSI: Clinical and Laboratory Standards Institute; MS-CoNS: Methicillin-susceptible CoNS.

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**Authors’ contributions**

MN and ZS performed the tests, collected and analyzed the data, BA was advisor and contributor in writing and editing the manuscript, GR, performed the analysis of the data. MA designed the project and contributes in the whole steps of the projects. All authors read and approved the final manuscript.

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**Availability of supporting data**

The Additional files included Additional file 1: Tables S1 and S2.

**Ethical approval and consent to participate**

This study was approved by the ethics committee of the Hamadan University of Medical Sciences (ICode No: IR.UMSHA.REC.1396.827) and about the consent to participate is not applicable.

**Consent for publication**

It’s not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Additional file 1: Table S1. Primers used in this study. Table S2. The prevalence of mecA gene and types of hemolysins among MR-CoNS species.
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