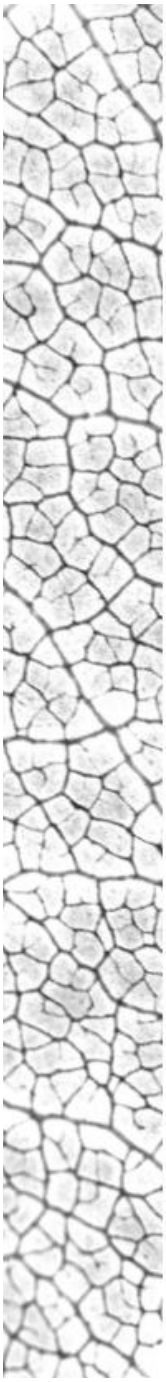

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Polymerase chain reaction detection of enterotoxins and hemolysin genes in coagulase-negative staphylococci isolated from patients with indwelling devices

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Abstract

Background and Aim: Coagulase-negative staphylococci are considered as nonpathogenic bacteria for long time as the components of normal flora in skin and mucosa, lately they become significant agents causing nosocomial infections, mainly with the presence of foreign bodies in the human. The study aimed to investigate the role of coagulase-negative staphylococci in human infections and determining the predominance genes of the virulence factors Materials and Methods: Three hundred clinical specimens were collected from out and in-patients undergoing catheter related infections and twenty specimens were collected from healthy hospital staff as a control of Al-Zahraa Teaching Hospital, Al-Sader Teaching Hospital and Al-Hakeem Hospital in Al-Najaf Al-Ashraf province. The specimens were included urine, blood, vaginal swabs, seminal fluid and wound swabs. The specimens were cultured on mannitol salt agar and the primary identification was depended on Gram stained and biochemical tests. Then finally identification with Vitek 2 system is done. Results: One hundred isolates were identified as coagulase-negative staphylococci (CoNS), Staphylococcus haemolyticus was identified as the most frequently isolated species in (53%), followed by Staphylococcus epidermidis (26%) and Staphylococcus hominis were recorded in (21%). Monoplex and multiplex PCR were used to explore the sea, seb, sec, sed, hla and hlb genes. PCR revealed that only (14%) of isolates had genes for enterotoxins expression. Of these, (92.86%) and (7.14%) was sea and seb genes respectively while sec and sed genes were not recorded. Conclusion: CoNS isolates obtained from clinical samples should be routinely treated and performed of antimicrobial susceptibility testing. The need for Coagulase-negative staphylococcal characterization and knowledge their pattern of antibiotic sensitivity to report as pathogenic bacteria causing human disease.

Keywords: Coagulase-negative staphylococci; Staphylococcus virulence factors; Enterotoxin.

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INTRODUCTION

Kloos and Bannerman were reorganized our information about the significantly of coagulase-negative staphylococci (CoNS) before twenty years ago, after that Pfaller and Herwaldt by their clinical, laboratory, and epidemiological characteristics (Becker et al., 2014). Coagulase-negative staphylococci are including more than 40 species and differentiated from related and closely virulent bacteria *Staphylococcus aureus* by their inability to produce free coagulase (Rogers et al., 2009). Members of coagulase negative staphylococci are gram positive cocci, non-motile, non-spore forming, unencapsulated, and most of them are

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facultative anaerobes (Pinna, 1999). Coagulase-negative staphylococci are commensal skin bacteria and become major pathogenic bacteria in nosocomial and opportunistic infections (Argemi, 2019), for long time they have considered as culture contaminants but now they have important role as pathogenic bacteria, approximately 55-75% nosocomial infection strains are methicillin resistant (Piette & Verschraegen, 2009). The persistent of CoNS may because have specific characteristics that enable them to survive in the host (Klingenberg, 2007). Coagulase negative staphylococci have several virulence factors but lacks that shared with the closely related species *Staphylococcus aureus*, biofilm formation is one of the important virulence factors which serve as the primary acting of immune evasion of CoNS, these bacteria are adroit by biofilm formation and this is a key for their pathogenesis particularly to catheter related infections (Marchant, 2013). Most CoNS are produce several exoenzymes such as lipases and proteases, which help to persistence of these organisms in the host and degrading the host tissue (Otto, 2004). Most species of CoNS including *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* were produce hemolysins which binds with the red blood cells resulting rapid release of internal molecules and lyses them with release of the free iron using by the bacteria (Enabulele, 2013). Staphylococcal enterotoxin (SE) also produced by CoNS were exoproteins established their ability to mitogens of T-cell, therefor, they called superantigens due to the directly binding to the major histocompatibility complex (MHC) class II and T-cell receptors (Veras, 2008). Family members of staphylococcal enterotoxins were divided into five types according to their antigenicity (*sea*, *seb*, *sec*, *sed*, *see*), also toxic shock syndrome toxin-1 (TSST-1) is a member of these family which stimulate large populations of T-cells receptors led to release massive cytokines that responsible of severe characteristics of TSST-1 (Soares Casaes Nunes, 2015). Bacteremia related with indwelling devices are infections most commonly caused by coagulase-negative staphylococci, these infections are hospital-acquired caused by strains transmitted among hospitalized patients (Johannes Huebner et al., 1991). The most significant species of CoNS which frequently cause infections in humans are *Staphylococcus epidermidis* (blood infections, catheter related infections, surgical wounds, osteomyelitis etc.), *Staphylococcus haemolyticus* (urinary tract infections, endocarditis, wounds, septicemia etc.), and *Staphylococcus saprophyticus* (septicemia and urinary tract infections). While, other CoNS including *Staphylococcus hominis*, *Staphylococcus cohnii*, *Staphylococcus simulans* and *Staphylococcus warneri* caused endocarditis and osteomyelitis (Cunha et al., 2004). CoNS species that colonized on the skin and mucous membranes in the humans and animals are less involved in clinically revealed infections, other species which found as food associated saprophytes (Becker et al., 2014). *S. epidermidis* are novobiocin susceptible species considered as a major cause of nosocomial infections specially bacteremia in patients with immunocompromises and indwelling foreign bodies, while *S. saprophyticus* isolated as novobiocin resistant species is most commonly cause of urogenital tract infections which infect the immunocompetent patients especially sexually active women and men (Von Eiff et al., 2001).

Antibiotics resistance of CoNS are determined by mobile genetic elements pool in humans and animals which led to multidrug resistance to aminoglycosides, macrolides, β-lactams, quinolones, and tetracyclines. Methicillin-resistant CoNS had *mec*A gene carried on staphylococcal chromosomal cassette *mec* (Bora, 2018). The resistance to oxacillin in coagulase free staphylococci increased in recent years, this is led to use of glycopeptide antibiotics for infections treatment. During 1990 increased resistance to glycopeptide antibiotics, the researchers suggesting which may be highly distributed in the community and hospitals (Bora, 2018). Acquisition of pathogenic genes which convert of commensal skin staphylococci into pathogenic bacteria, most species of CoNS are resistant to antibiotics due to the highly use of antibacterial drugs in hospitals which provided reservoir of resistant strains to antibiotics. Methicillin resistant CoNS are generated from genetic exchange between CoNS species and *S. aureus* (Deyno et al., 2018).

The study aimed to determine the molecular characterization for virulence factors of coagulase-negative staphylococci via the isolation and identification of these bacteria from different specimens, phenotypic determine of virulence factors by the according methods, and genotypic determine of virulence factors by polymerase chain reaction.

MATERIAL AND METHODS

Specimens Collection. In this study, three hundred clinical specimens were collected from out and inpatients of Al-Zahraa Hospital, Al-Sader Teaching Hospital and Al-Hakeem Hospital in Al-Najaf Al-Ashraf province/Iraq. The specimens were included, 100 specimens from urine, 60 from blood, 55 from high vaginal swabs (H.V.S), 35 from seminal fluid, and 50 swabs from wound. The patients how collected from them the specimens were suffering from urinary tract infections, bacteremia, vaginal infections, prostatitis, and pyogenic infections called post-operative wound infections and burn wound infections. In addition to,

twenty specimens were collected from healthy medical staff regarded as control.

Specimens processing. The collected specimens were cultured on mannitol salt agar, a selective and deferential medium for isolation and identification of staphylococci. The plates were incubated at 37°C for 24hours, then a single colony was subcultured on trypticase soya agar for the preservation and further processing such as biochemical tests which confirmed the identification of isolates including, catalase test, coagulase test, modified oxidase test, voges-proskauer test, bacitracin diagnostic test, novobiocin diagnostic test, and Vitek-2 for identification.

Hemolysis Determination. α-hemolysin was occurred on blood agar base with 5% of rabbit erythrocytes, the plates were incubated for 24hours at 37°C, then the positive samples showed a wide zone of complete hemolysis with blurred edges. β-hemolysin resulted on blood agar base with 5% of sheep erythrocytes. All the plates were incubated at 37°C for 24hours and then overnight in refrigerator, the positive strains showed incomplete hemolysis zone with sharp edges (Süheyla TÜRKYILMAZ, 2005).

Extraction and Isolation of DNA. The genomic DNA was isolated in method that described by (Arciola et al. 2001).

Estimation of DNA Concentration. Add 5µL of DNA sample to 995µL of distilled water mixed well, determined the concentration and purity of DNA by reading the optical density at 260 and 280 nm in spectrophotometer (Williams & McCarrey, 2007).

Polymerase Chain Reaction (PCR) Assay. The technique of PCR was done in monoplex and multiplex designs for amplify different fragments of genes under study to detecting the staphylococcal virulence factor genes (Table 1).

Table 1: Determining virulence genes and encoding properties.

Genes	Encodes
hla	α-Hemolysin
hlb	β-Hemolysin
sea	Enterotoxins A
seb	Enterotoxins B
sec	Enterotoxins C
sed	Enterotoxins D

Selection of PCR Primers. The selected oligonucleotide primers were used in conventional PCR amplification (Table 2), before that they liquified in lyophilized product followed spinning briefly with TE buffer depending on manufacturer instruction to form stock solution. Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer.

Table 2: The primers and their sequences used in conventional PCR for detection of CoNS virulence factors.

Target Gene	Primer Type	DNA sequence (5´-3´)	Product Size (bp)	References
hla	Hla	F: GGT TTA GCC TGG CCT TC R: CAT CAC GAA CTC GTT CG	550	(Singh et al., 2011)
hlb	Hlb	F: GCC AAA GCC GAA TCT AAG R: GCG ATA TAC ATC CCA TGG C	840	(Singh et al., 2011)
sea	Sea	F: TTG GAA ACG GTT AAA ACG AA R: GAA CCT TCC CAT CAA AAA CA	120	(Cunha, 2006)
seb	Seb	F: TCG CAT CAA ACT GAC AAA CG R: GCA GGT ACT CTA TAA GTG CC	478	(Cunha, 2006)
sec	Sec	F: GAC ATA AAA GCT AGG AAT TT R: AAA TCG GAT TAA CAT TAT CC	257	(Cunha, 2006)
sed	Sed	F: CTA GTT TGG TAA TAT CTC CT R: TAA TGC TAT ATC TTA TAG GG	317	(Cunha, 2006)

PCR Cycling Conditions. Mixture of PCR was set up in a total volume of 30µL included 15µL of PCR premix, 2µL of primer and 5µL of purified DNA have been used, then the volume was completed with 6µL of sterile deionized distilled water and vortexed. The negative control was contained distilled water instead of template DNA. The PCR tubes were centrifuged briefly for mix and placed into thermocycler PCR instrument where DNA was amplified as indicating in below (Tables 3-5).

Table 3: Program used to amplify the *sea*, *seb*, *sec* and *sed* genes**.**

Stage	Temperature (time)

Initial denaturation	94ºC for 2min	
Denaturation	94ºC for 2min	40 cycle
Annealing	55˚C for 2min	
Extension	72˚C for 1min	
Final extension	72˚C for 7min	

Table 4. Program used to amplify the *hla* gene.

Stage	Temperature (time)	
Initial denaturation	94ºC for 5min	
Denaturation	94ºC for 30sec	35 cycle
Annealing	53˚C for 30sec	
Extension	72˚C for 1min	
Final extension	72˚C for 5min	

Table 5. Program used to amplify the *hlb* gene.

Stage	Temperature (time)	
Initial denaturation	94ºC for 5min	
Denaturation	94ºC for 30sec	35 cycle
Annealing	62˚C for 30sec	
Extension	72˚C for 1min	
Final extension	72˚C for 5min	

Agarose Gel Electrophoresis. 1.7% agarose gel was prepared according to (Mainiatis & Sambrook, 1982), the gel was run for about an hour and a half and visualized.

RESULTS

Isolation of Coagulase Negative Staphylococci. In this study, from three hundred specimens were examined, only one hundred bacterial isolates grown on mannitol salt agar as selective medium with white colonies appeared round, smooth, raised, mucoid and glistening (Figure 1). then the isolates which suspected as CoNS were more purified by streaking method and selected according to their morphology of colony at first then further characterized by biochemical tests. On the other hand, twenty control isolates from nasal samples of healthy personnel, all of them grown on mannitol salt agar suspected as CoNS.

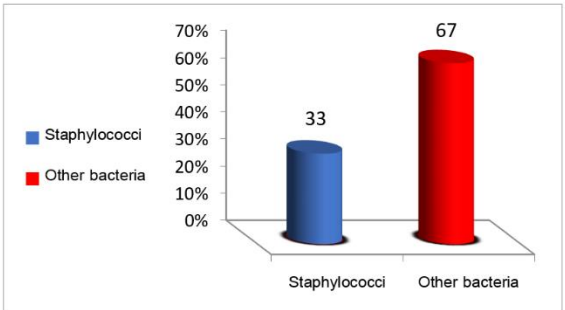

Figure 1: The percentage of staphylococci comparison with other bacteria

The results obtained from identification by Vite2 system revealed that all one hundred isolates identified as staphylococci, and the isolates were distribution into three species 53 isolates as *S. haemolyticus*, 26 isolates as *S. epidermidis*, and 21 isolates were identified as *S. hominis*. The most common species isolated was *S. haemolyticus* (53%), then *S. epidermidis* was the second common species in (26%). All the one hundred pathogen and twenty control suspected isolates were undergoing to biochemical tests (Table 6), the results showed catalase positive, coagulase negative, oxidase negative, voges-proskauer positive, sensitive to Novobiocin and resistance to Bacitracin antibiotic disc (Figure 2).

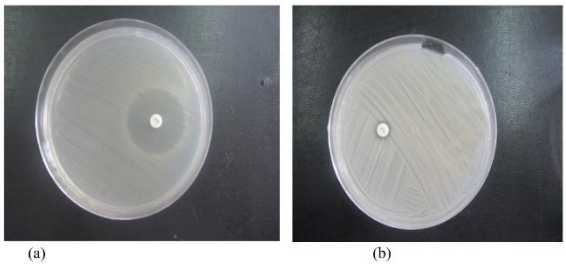

Figure 2: (a) resistance to Bacitracin by staphylococci, (b) sensitive to Bacitracin by micrococci

Table 6. Biochemical tests of pathogenic and control isolates of CoN-Staphylococci**.**

Species / Tests	S. haemolyticus		S. epidermidis		S. hominis	
	Pathogen	Control	Pathogen	Control	Pathogen	Control
Catalase	+	+	+	+	+	+
Coagulase	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	+
Novobiocin	sensitive	sensitive	sensitive	sensitive	sensitive	sensitive
Bacitracin	resistance	resistance	resistance	resistance	resistance	resistance

In this study, forty-seven isolates were obtained from urine specimens of patients with urinary tract infections, 28 were isolated from blood specimens of patients with bacteremia, 12 from high vaginal swabs, 13 isolates from seminal fluid from prostatitis males and no isolates were from wound infection (Table 7). The majority CoNS isolates were collected from female patients 61%, versus 39% of isolates were collected from male.

Table 7. Distribution of bacterial isolates with clinical specimens.

No.	Specimens	Total No. of Specimens	Total No. of Bacterial Isolates	Male	Female
1	Urine	100	47	9	38
2	Blood	60	28	17	11
3	H.V. S	55	12	0	12
4	Seminal fluid	35	13	13	0
5	Wound	50	0	0	0
6	Total	300	100	39	61

The effect of CoNS isolates on blood were tested on the two types of blood (rabbit and sheep blood) (Figure 3), and the identification of each hemolysin was achieved according to the shape of blood around of each bacterial colony, which corresponding with study of Akinjogunla and Enabulele (Akinjogunla & Enabulele, 2010). They identified by the presence of diffuse green color (α-hemolysis) or clear zone (β-hemolysis) around the colonies.

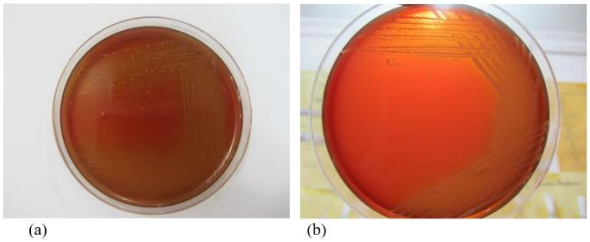

Figure 3: (a) alpha-hemolysin produced by CoNS on rabbit blood agar, (b) beta-hemolysin produced by CoNS on sheep blood agar

The study was included attempt to detect the predominant of genes encoding staphylococcal enterotoxins SEA, SEB, SEC and SED in CoNS isolates from patients with catheter related infections. The results showed that only 14 isolates (14%) had genes for enterotoxins expression, and the type *sea* was more frequently since it is found in 13 (92.86%) isolates, while *seb* appear in 1 (7.14%) isolates, in contrast, *sec* and *sed* genes were not be recorded (Figure 4)**.** The results of enterotoxin genes were observed in 10 isolates of *S. haemolyticus*, all of them were *sea* type only, on the other hand, two isolates of *S. epidermidis* showed carrying *sea* gene and one

isolate showed carrying *sea* and *seb* genes; while, no genes were detected in *S. hominis* isolates (Table 8).

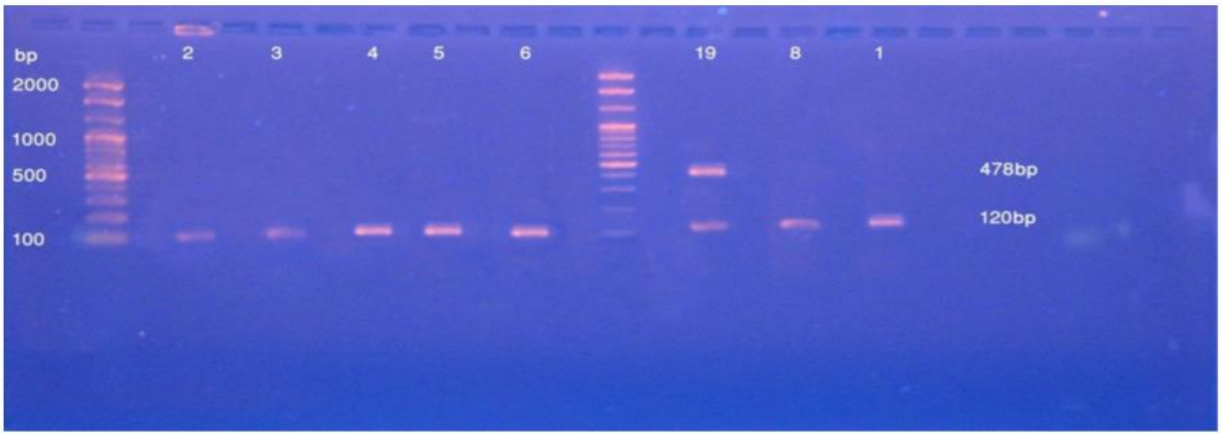

Figure 4: PCR amplification of enterotoxin genes (sea=120bp and seb=478bp) of CoNS species: L-DNA ladder 100bp, lanes (2,3,4,5,6) positive isolates of S. haemolyticus for sea gene, lanes (1,8,19) positive isolates for sea gene and lane 19 positive for seb gene of S. epidermidis

Table 8. Distribution of enterotoxin genes among coagulase-negative staphylococcal isolates.

CoNS	Enterotoxin genes				Total
	sea	seb	sec	sed	
S. haemolyticus	10	0	0	0	10
S. epidermidis	3	1	0	0	4
S. hominis	0	0	0	0	0
Total	13	1	0	0	14

The result of study showed that 47% of CoNS isolates had hla gene and 41% contain hlb gene. The presence of hla gene was an absolute predictor of phenotypic α-hemolysin producers, thus, 47% of hla+ strains were hemolysis of blood. Similarly, 41% of strains possessed hlb gene (Figure 5).

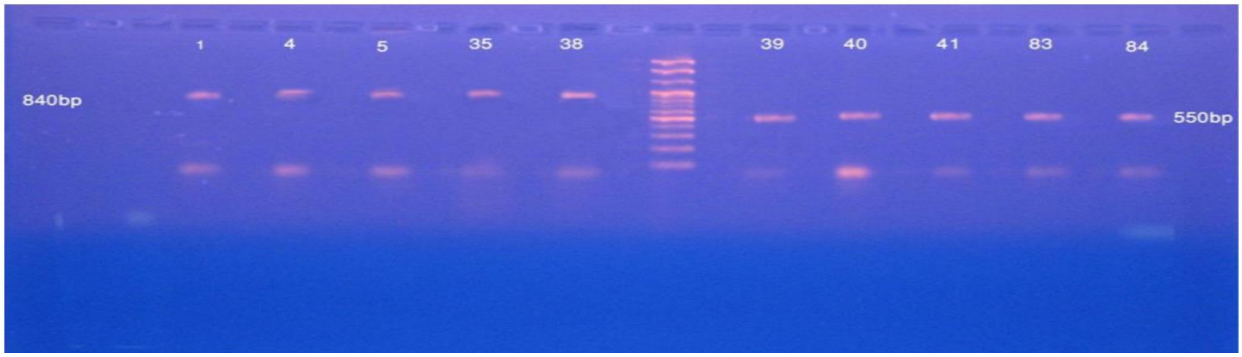

Figure 5: PCR amplification of hla (550bp) and hlb (840bp) genes of CoNS species: L-DNA ladder 100bp, lanes (1,39,40) positive isolates of *S. epidermidis*, lanes (4,5,35,38,41) positive isolates of *S. haemolyticus*, lanes (83,84) positive isolates of *S. hominis*

Lysis of red blood cells by CoNS are mainly mediated by the hemolysins referred to as alpha (α), beta (β) and delta (δ) toxins. The α toxin was encoded by the *hla* gene (Bubeck Wardenburg et al., 2007). The α toxin which produced by majority of *Staphylococcus* isolates is active against a wide range of mammalian cells and against rabbit erythrocytes (Husmann, 2009). Firm strains of *Staphylococcus* also produce beta (β) toxin, sphingomyelinase encoded by the *hlb* gene. β toxin is

extremely hemolytic against sheep erythrocytes but not against rabbit erythrocytes (Dinges et al., 2000).

DISCUSSIONS

Coagulase negative staphylococci are most resident microflora in the human body and could not cause harmful to the healthy individuals, on the other hands, they cause serious infections in compromised persons, especially in patients with indwelling devices such as catheters (Uyanik, 2014). In this study, only 100 bacterial isolates from 300 specimens were grown on mannitol salt agar, study of (Shittu, 2006), founded from 84 mannitol salt positive staphylococcal isolates were obtained from 240 nasal samples, from which 15 isolates were CoNS. Mannitol salt agar is a selective medium because the high concentration of salt about 7.5% which inhibits the growth of greatest bacteria, on the other hand, the bacteria that tolerant the salt can grow and proliferate on these media. Also, this medium contain mannitol, which serves as a differential agents (Davis et al., 2006). The results show that the one hundred bacterial isolates characterized into three species (S. haemolyticus, S. epidermidis, S. hominis) and the most common species was S. haemolyticus, the similar results were recorded in many studies of de De (Paulis, 2003), (Wilkie, 2012) they reported that majority number of isolates was S. haemolyticus. On the hand, (Singh et al., 2008) and (Asangi et al., 2011) showed on their results that S. epidermidis was the second common species similar with my results.

Staphylococcus haemolyticus is the most commonly species of bacteria which play significant roles in hospital acquired infections especially in patients with underlying disease, such as used of prosthetic devices, surgical infections, diabetes patients, and persons with dialysis (Krzyminska et al., 2012).

The study show majority of CoNS isolates were collected from female compared with male, this findings were correlated with the study of (Kumari, 2001), who reported that most CoNS isolates from females 54.1% versus 45.90% from males, while the study of (Souvenir, 1998) founded that 58% of isolates obtained from male and 42% from female isolated from blood cultures.

Bloodstream infection (BSI) mainly caused by coagulase-negative staphylococci which are the most frequently isolated pathogenic bacteria in blood stream, also these bacteria are contaminants the blood cultures, therefore in our study it is important to discriminate between bloodstream infections and contaminations (Elzi et al., 2012). (Dzen, 2007) reported to that CoNS was the third cause of bacteremia after Staphylococcus aureus and Escherichia coli. The administration of drugs by using of central venous catheters has become almost required in patients with serious infections, and coagulase negative staphylococci are still the most frequent pathogens (Schille, 2000).

Coagulase negative Staphylococci are considered of little significance as a cause of urinary tract infections (UTI), these bacteria regarded as urine contaminants rather than cause serious urinary tract infections (Dangel, 2011). There are several studies showed that rectal, vaginal, and urethral colonization of S. saprophyticus was associated with UTI caused by CoNS, This bacteria also reside in the urinary tract and bladder of sexually active females (Rall, 2010).

These results were agreement with the study of (Borelli, 2011) who reported that seventeen (26.2%) of 65 CoNS strains had genes for enterotoxins production, and the type sea was more frequently, it was found in (18.5%) of strains, followed by sec in three and seb in two strains, whereas the type sed gene was not recorded. The result study was disagreement with the study of (Abe, 2012) they found that the enterotoxin genes (SEA, SEC and SED) were not detected in any of the staphylococcal isolates. (Dobranić, 2013) they are suggesting that clinical CoNS isolates does not carry super antigenic toxin genes. In addition, the study of (Bertelloni, 2015) found that no one of CoNS isolates were carried enterotoxin genes. Food poisoning were caused by staphylococci occurred by absorption of contaminated food containing enterotoxins excreted by these bacteria, enterotoxins (SEs) are divided into five types according to antigenicity (sea, seb, sec, sed, see). Staphylococcal enterotoxins (SEs) and toxic shock syndrome toxine-1 that designated as SE-like toxins because they lack in emetic activity. Although SE produced by Staphylococcus aureus, several studies shows the ability of CoNS to production of SE in the last years.

Conclusion

We conclude that coagulase-negative staphylococci (CoNS) have become predominant in catheter related infections because of the combination of increased use of indwelling devices. S. haemolyticus, S. epidermidis and S. hominis are the species were identified in CoNS, S. haemolyticus are the most common species. The surveillance of nasal colonization with slime-forming oxacillin-resistant CoNS in health-care workers

might be helpful in breaking the epidemiological chain of hospital-acquired infections. The determinant of virulence factor genes in CoNS isolates, indicated that the agents may be developed the new virulence factors and their pathogenicity.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

SA drafted the manuscript, compiled information from the literature, and designed the figures and tables. WA reviewed and edited the manuscript.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

None.

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