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Slime Production and Antimicrobial Resistance in Coagulase -negative Staphylococci Isolated from Breast-milk of Lactating Mothers

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Authors' contributions

This work was carried out in collaboration between both authors. Author KTM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AEB and KTM managed the analyses of the study and they managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Background: The slime that produces by bacteria are responsible for many chronic infections and it's not easy to treat because they showing more resistance to antibiotic. Clearly it's the main virulence factors that determined pathogenicity of coagulase-negative staphylococci (CoNS), and found to be slime production and their effect on resistance of antibiotic.
Objective: This study was conducted to evaluate slime production and antibiotic resistance in CoNS isolated from breast-milk of lactating mothers
Study Design: Point prevalence cross-sectional study.
Place and Duration of Study: Breast milk samples were collected from 200 patients suffering from mastitis and 106 lactating women as control who visited the center of breast examination in hospital Al- Sadder –in Najaf- Iraq, during the period from July/ 2015 to Jun/ 2016).
Methodology: A total of 88 strains of coagulase negative Staphylococci (CoNS) isolated from breast milk by culturing it on Baired parker agar and mannitol salt agar then characterized and

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subjected to species level by using biochemical tests and Vitek-2 system, slime detected by Congo red agar method (CRA) and *ica*D gene detection by PCR , with antibiotic resistance profile using Vitek -2 system (bioMérieux, France**),** AST-GP580 Gram positive susceptibility cards

Results: Slime production was detected in most isolates (86/88) 97.72% phenotypically, however, 62 isolates were typed to species level (40 isolates of *S.epidermdis*; 12 isolates of *S.hominis*; and 10 isolates of *S.haemolyticus*), all these strains have the slime production gene (*icaD* gene) and showed different antibiotic resistance profile.

Conclusion: This study showed a good correlation of presence of *icaD* gene with the antibiotic resistant.

Keywords: Breast milk; mastitis; coagulase negative staphylococci; slime production; PCR; antibiotic resistance.

1. INTRODUCTION

Coagulase-negative staphylococci (CoNS) are present on external areas of the body as part of the normal skin flora, they have historically been considered to be non-pathogenic In terms of history CoNS have been considered to be a secondary pathogen or minor pathogen of mammary gland [1]. CoNS are taken as to be opportunistic organism of breast skin. Virulence factors are not very clear established and documented in CoNS compared to *S. aureus, in* CoNS no major toxins or virulence factors have been found and it is clear that development and persistence of coagulase-negative staph diseases must be due to alternative mechanisms [2].

Various *in vivo* and *in vitro* studies have reported that staphylococci yield a viscous extracellular material (slime) consisting of carbohydrate and protein that enables these bacteria to adhere to and colonize flat surfaces of the body [3] and [4] While slime production facilitates adherence on to surfaces, enzymes such as β-glucuronidase and phospholipase remote colonization [5].

The initial bacterial monolayer adhering to polymeric surfaces is converted to a typical biofilm consisting of bacteria plus an extracellular slime substance [6]. Adhesion, bacterial proliferation and slime production increase antibiotic resistance, since drugs may not be able to reach bacteria kept in rein in biofilm. Molecular studies have shown that late phases of adherence, in which organisms first adhere to each other and then elaborate a biofilm, are mediated by polysaccharide intercellular adhesion (PIA), which is synthesized by products of the *icaADBC* operon [7] and [8]. CoNS have been reported to be resistant to many antibiotics [3,9] and [10]. In another group of studies, slime production increases the severity and potency of infections and protects bacteria from the effect of antibiotics, resulting in antimicrobial resistance [11,12,13,14,15,16,17] and [18].

The present study, therefore, aimed to compare the slime production rates phenotypically and geneotypically at the same time compare those detected genotypically with the antibiotic resistance rates patterns of isolates.

2. MATERIALS AND METHODS

2.1 Patients and Samples

Breast milk samples were collected from 200 patients suffering from mastitis and 106 lactating women as control who visited the center of breast examination in hospital Al- Sadder –in Najaf- Iraq, during the period from July/ 2015 to Jun/ 2016). Milk samples were collected aseptically. The nipple and areola of the affected breast was cleaned with normal saline solution; the woman was given non-sterile surgical gloves and asked to manually express several drops of milk, which were discarded before collecting the specimen directly into the test tube [19], then transferred to the lab in cold condition. Samples cultured on baired parker agar for isolation of staphylococci (the colony number <4 cfu/ml^{-1}) [20]. CoNS were identified by biochemical tests [21], and by vitek-2 system to species level as well.

2.2 Phenotypic Slime Production Method

The ability of slime production of all staphylococcal isolates was evaluated using Congo red agar method phenotypically as described by Freeman et al. [22]. Congo red medium contained the following agents: 37 g/l brain heart infusion broth, 50 g/l sucrose, 10 g/l agar and 0.8 g/l Congo red dye. Bacterial strains (*S. aureus* and *S. epidermidis*) were cultured

onto Congo red agar (CRA). The assay plates were incubated at 37℃ for 24 hrs. All plates were examined in terms of colour change after 24 to 48 hrs. of incubation. Three criteria for slime synthesis were considered depending on the appearance of the colonies; strong positive (dry black), intermediate (smooth black), and negative slime producers (dry red, smooth red) [22].

2.3 Antibiotic Susceptibility Method by Vitek – 2 System

Antibiotic sensitivity testing was performed for isolates that were typed to species level, the isolates were subjected to antibiotic susceptibility testing by using the Vitek -2 system (bioMérieux, France), AST-GP580 gram positive susceptibility cards. The isolates that tested for antibiotic susceptibility were only those that were diagnosed for genus and species, which included *S. epidermidis* (40 strains), *S. haemolyticus* (10 strains), *and S.hominis* (12 strains) (about 50 strains from patients with mastitis and only12 strains from healthy women). Antibiotics used included cefoxitin screen (used to confirm the presence of MRSA and detect low level methicillin resistance), benzyl penicillin, oxacillin, mupirocin, clindamycin, inducible clindamycin resistance, tetracycline, as well as aminoglycosides (gentamicin, tobramycin), quinolones (levofloxacin, moxifloxacin), and glycopeptides (teicoplanin, vancomycin) [23].

2.4 Molecular Detection of Slime

2.4.1 DNA extraction

DNA extraction was carried out according to the genomic DNA purification kit supplemented by manufactured company (Geneaid).

2.4.2 PCR detection of *ica*D gene

The primer specific for amplification of slime gene (*ica* D) was: forward primer (ATGGTCAAGCCCAGACAGAG) and reverse primer (TGTCACGACCTTTCTTATATTTTTGA) were designed by Yang et al. [24] PCR condition used to amplify Slime gene involved the following: each 20 ML of PCR reaction contained (5µl of DNA template, 10pmol of forward primer, 10 p mol reveres primer, and 12.5 µl master mix, then volume was completed with molecular grade water. The PCR amplification product expected to be 301 base pair (bp) was visualized by electrophoresis on 2 % agarose gel, at 100 volt and 80 AM for 1hr.The size of amplicon was determined in comparison to the 100bp ladder (Promega, USA).

3. RESULTS

3.1 Distribution of Coagulase Negative Staphylococci

A total of 88 isolates of CoNS isolated from breast milk of lactating women (66 patients with mastitis and 22 healthy as control) included in the study as shown in Table 1.

The distribution of CoNS as follows: *S .epidermdis* 40 strains, followed by *S.hominis* 12 strains, *S.haemolyticus*10 strains, and Other coagulase negative staphylococci 24 strains recovered for patients suffering from mastitis and control women.

Only 62 isolates were typed to species level and were subjected to antibiotic susceptibility testing.

3.2 Phenotypic Slime Production Method

This test was conducted on all CoNS isolates (both typable and non-typable) the results of biofilm production by CoNS using CRA assay (Table 2) on the basis of [22]. The results showed that 97.72% of the isolates were positive for biofilm production. Among 88 isolates of CoNS, 22 strains were strong biofilm producer (dry black Fig. 1C), 64 were intermediate producer (smooth black), while only 2 isolates were negative (dry red or smooth red) as shown in Fig. 1A and B.

Table 1. Distribution of CoNS recovered from patients and healthy women

CoNS isolates	Mastitis samples (%)	Control (%)
S. epidermdis	45.45% (30/66)	45.45% (10/22)
S. haemolyticus	15.6% (10/66)	0
S. hominis	15.6% (10/66)	9.09% (2/22)
Others	24.24% (16/66)	15.6% (8/22)
Total	100% (66/66)	100% (22/22)

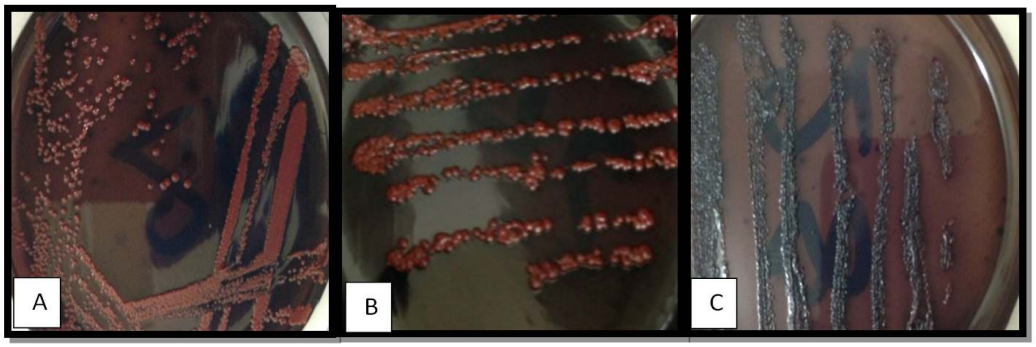

Fig. 1. CoNS isolates biofilm production assay on Congo red agar; A- strong positive (dry Black colonies); B- negative (smooth red); C-negative (dry red)

3.3 Antibiotic Susceptibility Testing

It was performed by vitiek-2 method. The antibiotic susceptibility of isolates as appeared in Fig. 2 was done only for the typable species of CoNs which were 62 strains comprising (50 from patients with infectious mastitis and 12 from control group) which also representing: (*S. epidermidis* 40, *S. haemolyticus* 10, and *S. hominis* 12). It is noticed from (Fig. 2) that Most of CoNS (56/62) isolates were resistant to penicillin G, Cefoxitin, oxacillin, 90.32%(12/62) gentamycin 38% tetracycline 19.35%, Sulfamethoxazole 9.67% (6/62), Ticoplanin 7%, Rifampicin 6.45% (4/62), and Nitrofurantoin (3.3%). While Moxifloxacin, Linezolid, Vancomycin, and Tigecyclin showed full of activity (100%) against different species of the CoNS isolates.

3.4 Molecular Detection of Slime Gene

The results in Figs. 3, 4, 5, and 6 showed that all isolates have *ica*D gene 88/88(100%).

3.5 Phenotypic Versus Genotypic Study of Different Strains of CoNS Isolated From Mastitis and Control Groups

When the results of phenotypic (Congo-Red) study of different CoNS isolates were compared with that of genotypic (*icaD*) study for the same isolates Table 2, It was seen that all the isolates were compatible in both tests except two out of the twenty six (2/26) of the other (Non-typable) CoNS isolates.

3.6 Relationship between Antibiotic Resistance and *icaD* Gene for the Typable Species

The study appeared that all isolates that showed resistant to different antibiotic were positive for detection of *ica*D by PCR technique which thought to be responsible for slime then biofilm production as in Table 3. Out of 62 typable CoNS isolates, 56 were resistant to penicillin, Oxacillin and Cefoxitin. Moreover, all the typable CoNS surveyed strains were positive for the presence of *icaD* gene and Congo red test (62/62).

Gentamycin, Rifampicin and Ticoplanin showed low level of resistance (4/62) for each while Erythromycin (24/62), Inducible clindamycin resistance and Nitrofurantoin showed lower level of resistant (2/62) in spite they have slime and *icaD* gene 100% (62/62) for all, but Tetracycline, (12/62), Trimethoprim-sulfamethoxazole (6/62) resistance and are having full spectrum of slime and *icaD* gene (100%). Only Tobramycin, Levofloxacin, Moxifloxacin, linezolid, Tigecycline, Monoxycarbolic acid, and vancomycin showed full spectrum of susceptibility 100%.

Table 2. Phenotypic and genotypic detection of slime production ability of Different strains of CoNS isolated from patients with mastitis and control group

Coagulase –negative staphylococci isolates	Phenotype (CRA) N=88	Genotype *icaD* N=88
S. epidermidis	(40/40)100%	(40/40)100%
S. heamolyticus	(10/10)100%	(10/10)100%
S. hominis	(12/12)100%	(12/12)100%
Others	(24/26)92.30%	(26/26)100%
Total	(86/88)97.72%	(88/88)100%

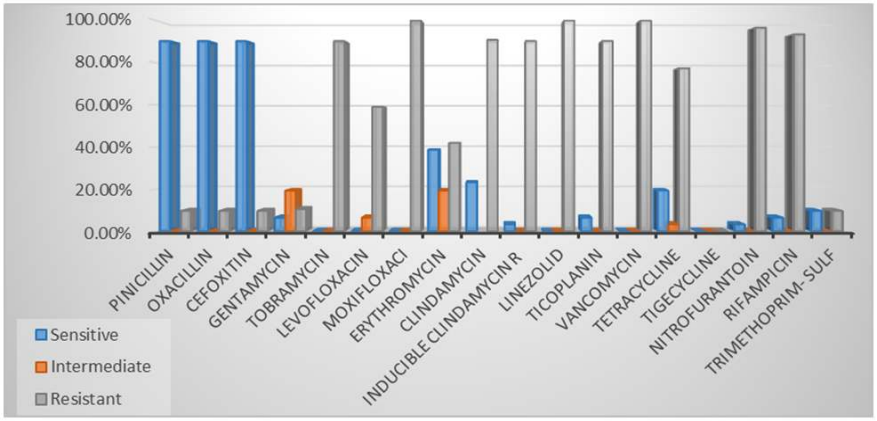

Fig. 2. Antibiotic sensitivity pattern of 62 coagulase negative staphylococci isolates

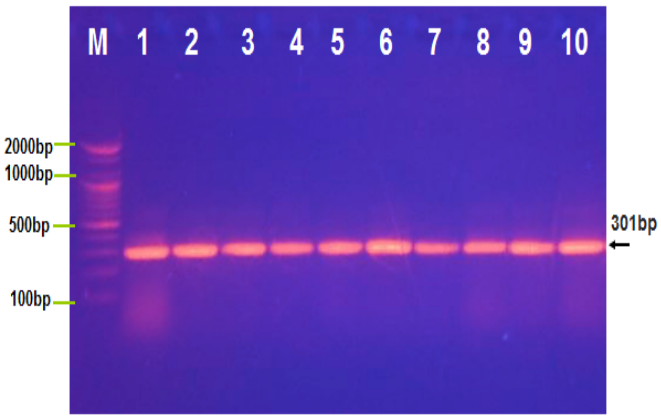

Fig. 3. Agarose gel electrophoresis image showing the PCR results of *icaD* gene in *Staphylococcus epidermidis* isolates. Where M: Marker (100-2000bp), lanes (1-10) positive *icaD* gene at (301bp) PCR product size

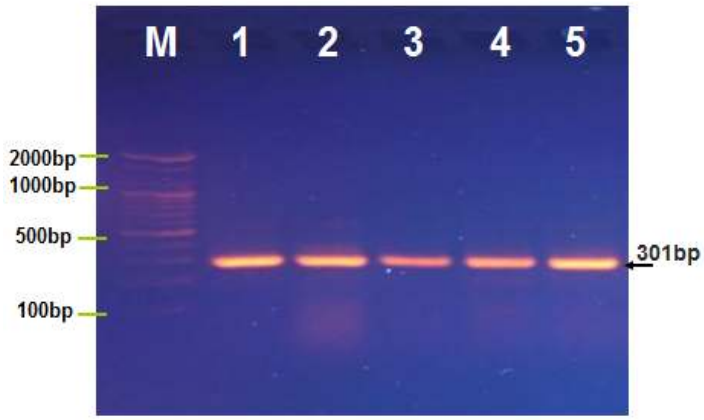

Fig. 4. Agarose gel electrophoresis image showing the PCR product of *icaD* gene in *Staphylococcus haemolyticus* isolates. Where M: Marker (100-2000bp), lane (1-5) positive *icaD* gene at (301bp) PCR product size

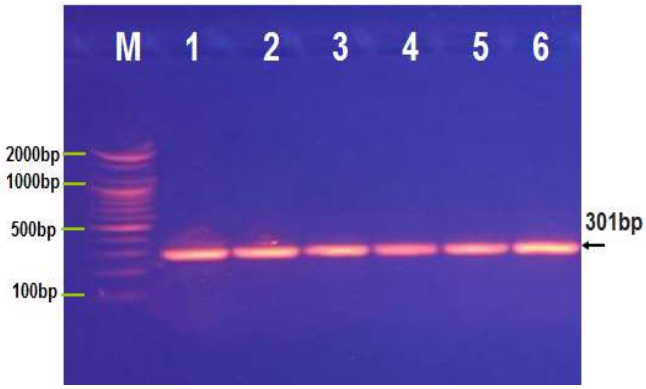

Fig. 5. Agarose gel electrophoresis image showing the PCR product of *icaD* gene in *Staphylococcus hominis* isolates. Where M: Marker (100-2000bp), lane (1-6) positive *icaD* gene at (301bp) PCR product size

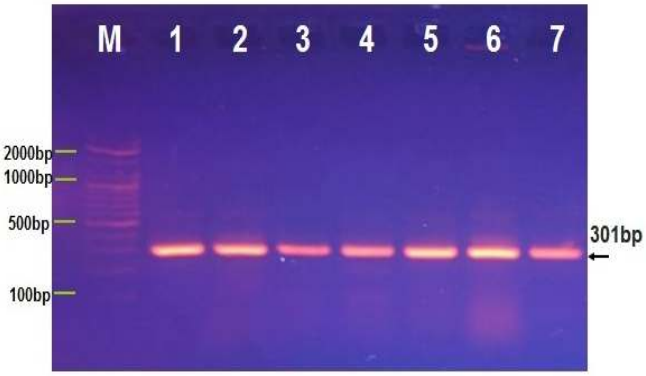

Fig. 6. Agarose gel electrophoresis image showing the PCR product of *icaD* gene in other coagulase negative isolates. Where M: Marker (100-2000bp), lane (1-7) positive *icaD* gene at (301bp) PCR product size

Table 3. Relationship between antibiotic resistance and *icaD* gene for the typable CoNS species

Antibiotics	Resistant	*icaD*
Penicillin	(56/62)90.32%	(62/62)100%
Oxacillin	(56/62)90.32%	(62/62)100%
Cefoxitin	(56/62)90.32%	(62/62)100%
Gentamycin	(4/62) 6.45%	(62/62)100%
Tobramycin	0	(62/62)100%
Levofloxacin	0	(62/62)100%
Moxifloxacin	0	(62/62)100%
Erythromycin	(24/62) 38.70%	(62/62)100%
Clindamycin	(2/6) 23.22%	(62/62)100%
Inducible clindamycin- resistance	(2/62) 3.22%	(62/62)(100**%**
Linezolid	0	(62/62)100%
Ticoplanin	(4/62) 6.45%	(62/62)100%
Vancomycin	0	(62/62)100%
Tetracycline	(12/62) 19.35%	(62/62)100%
Tigecycline	0	(62/62)100%
Nitrofurantoin	(2/62) 3.22%	(62/62)100%
Rifampicin	(4/62) 6.45%	(62/62)100%
Trimethoprim-sulfa	(6/62) 9.67%	(62/62)100%

The distribution of CoNS is as follows: *S. epidermdis* 45.45% (16/66) (30/66) *S. haemolyticu*s 15.6% (10/66), *S. homin, Other* 24.24% (10/66) for mastitis case respectively and *S. epidermdis* (30/66) 45.45% *S. haemolyticu*s (10/22) *S, hominis* (2/22) 9.09% Others(8/22) 15.6% respectively as control.

4. DISCUSSION

4.1 Distribution of Isolates

When the CoNS isolate from both mastitis patients and control were reviewed, it was seen that *S. epidermdis* was more prevalent than other species among patients with mastitis and control women respectively, this finding was in agreement with what was found in report of Degaldo et al. [20] who could be isolated 200 *S. epidermidis* from 207 milk samples. Others [25] found that CoNS followed by *Streptococcus viridans* (*S. viridans*) bacteria are often isolated from milk samples. All these bacteria are normal flora of the breast environment through breastfeeding the predominance of S. *epidermidis* can be explained by the fact that this microorganism is normal flora on the breast skin and have the ability to become pathogenic for any cause as it was confirmed by the previous research just mentioned. In similar, many researches have suggested the inclusion of that type of bacteria (S. *epidermidis*) as means a factors of breast infection in various animal types [26,27] also just mentioned and also documented by Tena et al. [25].

4.2 Comparison of Phenotype (Slime) and Genotype of (*icaD*)

In this study we observed that all the clinical and majority of the control CoNS isolates gave positive results for molecular examination of *icaD* gene by PCR, the finding of traditional PCR test for the *icaD* gene and tests of phenotype were important studies on mastitis and develop diagnostic tests for biofilm-producing microorganisms. Previously, the results of CRA tests that indicated the finding of the *ica*D genes for strains of *S. epidermidis* that was isolated from mastitis and control, and the test of genotype was utilized as gold standard comparison [28,29] *ica*D loci existence in 100% of the mastitis *S.epidermidis* isolates determine the possible role as a virulence marker in causing and controlling of patient and control. Many authors have reported of the widespread of the *ica* loci in clinical isolates of *S*. *epidermidis* than in *S. saprophytic* strains [30,31,32,33,34].

Present results showed that the phenotypic results of Congo red assay is approximately similar to the result of genotypic result between (mastitis cases and control) for the CoNs expect in the other (nontybable) CoNs strians show that 92.30% of isolates positive for slime phenotype assay and 100% positive for genotype. The findings was higher than what was found by Satorres and Alcaráz [35,36] test in 41.3% and 38.5% of staphylococci isolates, respectively. and disagreed with Noha et al. [37] who suggested that there is no strong relation in the existence of the *ica ABCD* operon and in vitro biofilm formation in invasive, colonizing and contaminant *S. epidermidis* between most of the colonizer strains which tested in their research, have been biofilm producer were a positive for *ica.*

The finding of genes coding cell surface proteins may illustrate at least partially the high distribution of *S. epidermidis* in human milk, mammary areola and canals of each healthy and infected mother. The *icaD* gene was spread between isolates from breast infection cases (100%) and in control women (100%) A good correlation was seen between the phenotypic finding that has been got it by the use of Congo-red agar method, which detect potential for slime production, and all isolates that amplified for the gene *icaD* which is also gave a positive results by the phenotypic assay Table 2.

4.3 Antibiotic Susceptibility Test of CoNS

Most of CoNS (56/62) isolates were resistant to penicillin G, Cefoxitin, oxacillin, 90.32% . These results are going with the study of Ustulin and Cunha [38], who reported that all isolates (100%) of *S. haemolyticus* and *S. hominis* were resistant to oxacillin versus 82.3% of *S. epidermidis* isolates.

Study of Abd El Hafez et al. [39] found that 86-100% of CoNS isolated from neonatal infections were methicillin-resistant strains (MR-CoNS), moreover, these bacteria was connected with multiple resistance to other antibiotics penicillin and oxacillin resistance was appeared to be 86.8% and 29.7%, at follow but in this research it was 90.32%(56/62).

The main two methods are accountable for the insusceptibility of staph-bacteria to the β-lactam

antibiotics the 1st: β-lactamase enzyme production by the CoNS that damage these agents, and the 2nd: change of proteins found in the wall of the bacteria cell named (PBPs) [40].

The resistance of staphylococci to oxacillin might be mediated by gene *mec*A which is code to result an additional binding protein for penicillin, PBP2a or 2, which is mentioned either homogeneous or asymmetrically [41] PBP penicillin binding protein 2a has a small affinity for beta-lactam antibiotic, the homogenous resistance is really recognized with standard test methods, whereas the heterogeneous express is more complex to examination with a few tests, due to only a little part of the PBP2a is expressed in the resistant phenotype [42].

The resistance of CoNS to penicillin, oxacillin, cefoxitin (90.32%) was attributed to the fact that cefoxitin is considered to be an excellent inducer of mecA gene expression, therefore, staphylococci resistant to methicillin/oxacillin should be considered resistant to cefoxitin [43] The result study agreed with the study of Ustulin and Cunha, [38] and Secchi et al. [44] and they reported that the resistance of CoNS to cefoxitin was recorded in 83.3% and 100% (Fig. 2).

4.4 Antibiotic Resistant and *ica*D Presence

It is found from Table 3 that the clinical CoNS isolates had highly frequency of slime production and resistance to antibiotics, particularly *S. epidermidis*, *S.heamolyticus* and *S.hominis* isolates. Also, all staphylococci isolates that cultured from clinical samples were manifested resistance to different antibiotic (one or more agents). The results of current research might be useful to select antimicrobial agent which is suitable to successful therapy of diseases caused by staphylococci It could be concluded from this table that there is a good correlation between antibiotic resistant and slime formation and presence of *ica*D which is supported by the Figs. 3, 4, 5 and 6 . The results are similar to those reported by another researcher [45] who suggested that the findings of their studies showed that staphylococcus isolates containing biofilm character show more resistant to antimicrobial agents, and not easy treated [46], also the study of Ismail et al. [44] found a positive association between positive-slime production and resistance to antibiotics, while disagreed with the study of [47] who found no association between phenotypic and molecular tests for biofilm production, which also enhanced by other previous studies [28] and [29].

The production of an exopolysaccharide matrix may contribute to increase of cell life by delaying antimicrobial penetration. The impact of transport limitation on biofilm life has been analyzed in a number of studies, and this antibiotic resistance associated with biofilms has been confirmed to be due to bacterial growth and metabolic activity. It has been proposed that slow-growing and non-growing bacteria considerably contribute to a decrease biofilm formation ability and so susceptibility to antimicrobial agents increased. Oxygen availability is also postulated to contribute to the resistant of antibiotics of biofilms, since the non-attendance of oxygen was found to decrease the antibiotic activity of few antimicrobials [2].

5. CONCLUSION

This study showed a good correlation of presence of *icaD* gene with the antibiotic resistant *mecA* may be the gene responsible for methicillin resistant of staphylococcus.

CONSENT

It is not applicable.

ETHICAL CONDUCT OF THE STUDY

The study has been conducted in accordance with recommendations guiding obtained from the College of Medicine, Kufa University. The study did not involve biological material or genetically modified organisms. All the isolates involved in the study came from routine samples without any additional materials.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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