Molecular investigation of Mecgene Among coagulase negative Staphylococcus isolated from different cases

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Abstract: This study is aimed on investigating the coagulase negative Staphylococci (CONs) in human infections and mecA gene, that is responsible for some virulence factors. One hundred and fifty specimens were collected from different infection case theories, from the period February to June 2018, at AL-Diwanyah Teaching Hospital. The specimens included urine, ear swab, vaginal swab, pus, wound swab, skin carrier, nasal, nipple, stool and tracheal. The specimens were cultured on blood agar and mannitol salt agar. The identification was depended on gram stained and biochemical tests. Then final identification with APi staph system. Thirty three isolates were identified coagulase negative staphylococci (CONs) which included S.lentus and S.xylosus. Twenty isolates were highly resistant to methicillin, oxacillin and cefoxitin. The PCR were used to detected the mecA gene. The results showed that 20 isolates had mecA gene. Some virulence factors of CONs were detected including hemolysin - determined in 8 isolates, urease producing - determined in 13 isolates.

1. Introduction
Coagulase Negative Staphylococci (CONs) is one of the Causative agents of bacterial diseases. They are most important cause of life threatening infection in hospital [1]. The use of extended procedures, aggressive antibiotic drug protocols, and long hospital stays are the causes of development of CONs infections. CONs that are commonly benign commensally and cause disease. The isolated strains have increasingly become multi resistant to many antibiotic [2]. Percentage of methicillin, up to 80%, have been observed among CONs isolates from many infections. The resistance to beta lactam antibiotics are often more heterotypic than is the case with S. aureus [3]. Through some studies have shown a close correlation between oxacillin resistance and the mec A gene [4]. CONs are part of normal flora, the presence of antibiotics. Resistance to penicillin is mainly dependent on the expression of the mecAgene, which encodes penicillin binding protein transpeptidase with low affinity towards most of the semisynthetic penicillin [5]. The aim of this study was isolates and identify CONs from many clinical cases and determine CONs susceptibility to antibiotic and detection about mecA gene.

2. Materials and methods
2.1 Collection of Samples:
Collection of specimens were collected from 150 clinical cases and healthy carrier from Al Diwanyah teaching hospital, during the period from January 2018 to June 2018. The samples were collected and inoculated in enriched medium of brain heart infusion, incubated at 37 c for 18 – 24 hours. Morphological features’ on culture medium and biochemical tests with the use of APi staph System and classified [6].

This study is aimed on investigating the coagulase negative Staphylococci (CONs) in human infections and mecA gene, that is responsible for some virulence factors. One hundred and fifty specimens were collected from different infection case theories, from the period February to June 2018, at AL-Diwanyah Teaching Hospital. The specimens included urine, ear swab, vaginal swab, pus, wound swab, skin carrier, nasal, nipple, stool and tracheal. The specimens were cultured on blood agar and mannitol salt agar. The identification was depended on gram stained and biochemical tests. Then final identification with APi staph system. Thirty three isolates were identified coagulase negative staphylococci (CONs) which included S.lentus and S.xylosus. Twenty isolates were highly resistant to methicillin, oxacillin and cefoxitin. The PCR were used to detected the mecA gene. The results showed that 20 isolates had mecA gene. Some virulence factors of CONs were detected including hemolysin - determined in 8 isolates, urease producing - determined in 13 isolates.

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2.2 Growth on mannitol salt agar
The sample were inoculated on mannitol salt agar plate, and the plates were incubate at 37 c for 24 hours to isolate pure staphylococcal colonies and to detect the ability of each isolate to ferment mannitol sugar [7].

2.3 Biochemical tests
2.3.1 Catalase test
One to two drops of catalase test reagent (3%H2O2) were placed on a slide, a colony of tested bacterium was mixed with the reagent on the slide, positive results were indicated by air Bubbles formation [8].

2.3.2 Oxidase test:
A piece of filter paper placed in clean petri dish and 2-3 drops of oxidase reagent were added to the filter paper. The positive result was indicated by blue purple color formation within 10 - 15 seconds [9].

2.3.3 Coagulase test
A colony of tested bacteria was emulsified in 1 ml of diluted plasma 1:4 of physiological saline, the tubes incubated at 37c, then examined after 1-4 hours. The clot formation indicated a positive result, while the negative tubes re-examined after 24 hours [10].

2.3.4 Bacitracin sensitivity test
Streaked it all over the plate of Muller Hinton agar and then the bacitracin disc (0.04u) was placed and incubated at 37 c for 18-24 hours [11].

2.3.5 Novobiocin sensitivity test.
Using a novobiocin disc (5 mg/disc ) and in reading the results, with the appearance of colonies and the inhibition zone less than 16 millimeters indicated the resistance of the bacteria for this test [12].

2.3.6 Motility test
Semi solid mannitol medium was inoculated beading by stabbing and incubated at 37 c for 24 hours, spreading turbidity from the stab-line or turbidity throughout the medium was considered as positive result [13].

2.3.7 APi staph system test
The APistaph is the identification system for staphylococcus. This test is applied according to the supplied company instruction.

2.3.8 Detection of haemolysin production
Sheep blood agar plates prepared, were inoculated with the tested organism and the plates incubated at 27 C° for 24 hours. Then the plates were examined for observing the type of haemolysis.

2.3.9 Urease test
Using a sterile straight wire, tubes of urea medium were inoculated with a colony of tested bacterium, and incubated for 24-28 hours. Urease positive strain split off ammonia from the
urea which raises the pH and cause the phenol red, which is the pH indicator, turn to red pink [15].

2.4 Antimicrobial sensitivity test
2.4.1 Disk diffusion test.
The antibiotic discs (methicillin, oxacillin and cefoxitin) were placed on the surface of the medium and incubation was usually for 24 hours at 37 C° [16].

2.5 Polymerase chain reaction
PCR assay was performed for detection of mecA gene in coagulase negative staphylococci isolates

2.6 Genomic DNA extraction
Genomic DNA was extracted from bacterial isolates by using genome DNA mini bacteria kit.

2.7 Estimation of DNA extracts
The extracted DNA was checked by using nanodrop (thermo.USA) that measured DNA concentration (µg/ml) and checked the DNA purity by reading the absorbance at (260/280 nm) [17].

2.8 Detection of mecAgene by PCR method
MecA gene was amplified using mecA gene primers (mecA F: 5 _ AAAA TCGAT GG TAAAGGTT GGC_3) and mecA R: 5 AGTTCTGCAGTACCGGATTTGC_3), which were selected on the basics of the published nucleotide sequence. The PCR reaction mixture was prepared in a reaction mixture (50 Ml), contain tris_HCL (10 mmol/L), 0.2 mmol/L of each deoxynucleotide mgcl2 (1.5 mmol/L) 25 pmol of each primer and Taq polymerase (2.5 units)[18].
The PCR amplification consisted of one cycle at 94 C° for 3 minutes, followed by 30 cycle at 94 C° for 30 seconds, 55 C° for 30 seconds, 72 C° for 30 seconds, and then finally 72 C° for 3 minutes for the analysis of the mecA gene primers mecA R yielded a fragment of 532 bp [18].
The PCR product were examined using 2% agarose gel (Bio Basic (USA)) electrophoresis. The gel were stained with ethidium bromide (Bio Basic (USA) to detect of the fragment of mecA gene, and that as showed in results adder (Bioneer (Korea)) was used as a DNA molecular weight standard.

3. Results and Discussion
A total of 33 CONs strains, belonging to 2 species, were identified in this study (table 1). The species included S. lentus (20 isolates) and S. xylosus (13 isolates). S. lentus was the most common type of CONs. It was in agreement with other studies about predominant of S. lentus [19] and also quite agreement with [20], who found that 41 isolates belong to S. lentus. But these results are not in agreement with [21] the one who showed that S. epidermidis was the most common type of CONs. Resistance to bacitracin and novobiocin were done which confirmed that these isolates belong to the coagulase negative staphylococci [22].
Table 1. Results of the identification tests for isolates of coagulase negative staphylococci in this study

| No. | Sample       | Isolates | Catalase | Oxidase | Coagulase | Bacitracin | Novobiocin |
|-----|--------------|----------|----------|---------|-----------|------------|------------|
| 1   | Urine        | s. xylosus | +        | -       | -         | R          | R          |
| 2   | Uri          | s. xylosus | +        | -       | -         | R          | R          |
| 3   | Urine        | s. xylosus | +        | -       | -         | R          | R          |
| 4   | Ear swab     | s. xylosus | +        | -       | -         | R          | R          |
| 5   | Urine        | s. lentus  | +        | -       | -         | R          | R          |
| 6   | Urine        | s. lentus  | +        | -       | -         | R          | R          |
| 7   | Ear swab     | s. lentus  | +        | -       | -         | R          | R          |
| 8   | Vaginal swab | s. lentus  | +        | -       | -         | R          | R          |
| 9   | Wound swab   | s. lentus  | +        | -       | -         | R          | R          |
| 10  | Skin (carrier)| s. lentus  | +        | -       | -         | R          | R          |
| 11  | Skin (carrier)| s. lentus  | +        | -       | -         | R          | R          |
| 12  | Nasal swab   | s. lentus  | +        | -       | -         | R          | R          |
| 13  | Catheter     | s. lentus  | +        | -       | -         | R          | R          |
| 14  | Catheter     | s. lentus  | +        | -       | -         | R          | R          |
| 15  | Wound swab   | s. xylosus | +        | -       | -         | R          | R          |
| 16  | Catheter     | s. xylosus | +        | -       | -         | R          | R          |
| 17  | Catheter     | s. xylosus | +        | -       | -         | R          | R          |
| 18  | Catheter     | s. lentus  | +        | -       | -         | R          | R          |
| 19  | Catheter     | s. lentus  | +        | -       | -         | R          | R          |
| 20  | Catheter     | s. lentus  | +        | -       | -         | R          | R          |
| 21  | Urine        | s. lentus  | +        | -       | -         | R          | R          |
| 22  | Wound swab   | s. xylosus | +        | -       | -         | R          | R          |
| 23  | Catheter     | s. xylosus | +        | -       | -         | R          | R          |
In this study, the culture characterizes and bacterial identification that based on culture the colonies on blood agar plates appeared large, round, creamy colored, (figure 1) [23], and some colonies which appear β or α _ haemolysis [24]. All the isolates of CONs positive for catalase and negative to coagulase enzyme( table 1).The identification of isolates was confirmed by API Staph, (figure 2 ).

| Isolation Site | Species   | Catalase | Coagulase | Resistance |
|---------------|-----------|----------|-----------|------------|
| 24 Urine      | s. xylosus| +        | -         | R          |
| 25 Urine      | s. lentus | +        | -         | R          |
| 26 Urine      | s. lentus | +        | -         | R          |
| 27 Vaginal swab| s. lentus| +        | -         | R          |
| 28 Vaginal swab| s. lentus| +        | -         | R          |
| 29 Wound swab | s. lentus | +        | -         | R          |
| 30 Nasal swab | s. lentus | +        | -         | R          |
| 31 Nasal swab | s. xylosus| +        | -         | R          |
| 32 Pus        | s. xylosus| +        | -         | R          |
| 33 Pus        | s. xylosus| +        | -         | R          |

( + ) Positive , ( - ) negative , ( R ) resistance

**Figure 1.** growth of CONs on : (A) blood agar (B) mannitol salt agar
The result showed that 13 isolates (65%) of S. lentus and 7 isolates of S. xylosus were resistant to methicillin (table 2). These results was similar [25], who demonstrated that S. lentus has 67% resistance to methicillin, while in contrast with [20] high level of resistance (94%).

**Table 2.** Distribution of methicillin resistance CONs isolates detected in different clinical samples

| Samples         | No. S. lentus R. Methicillin | %  | No. S. xylosus R. Methicillin | %  |
|-----------------|------------------------------|----|-----------------------------|----|
| Urine           | 2                            | 15%| 3                           | 42.8%|
| Ear swab        | 1                            | 7.6%| 1                           | 14.25%|
| Vaginal swab    | 1                            | 7.6%|                            |    |
| Pus             | -                            |    |                            |    |
| Wound swab      | 1                            | 7.6%| 1                           | 14.2%|
| Skin (carrier)  | 2                            | 15%|                            |    |
| Nasal (carrier) | 1                            | 7.6%|                            |    |
3.1 Detection of some virulence factors in CONS
Hemolysin production in the present study was determined in 20 isolates CONS having hemolytic activity characteristics, which were methicillin resistant, the results showed that 8 isolates (40%) of CONS produced hemolysins, did not agree with the results [26], who found that CONS with detected hemolysins, determined 45.4% and this results was not in agreement with [27] the one who showed that 28.9% to the ability of CONS to produce Hemolysin were investigated in 20 isolates which were resistant to Methicillin and Oxacillin. So this study showed that 13 isolates (65%) were urease producer Table (3) [28].

Table 3. Distribution of some virulence factors among CONS isolated in this study

| No. | Sample         | Isolates | Urease | Haemolysin |
|-----|----------------|----------|--------|------------|
| 1   | Urine          | s. xylosus | +      | +          |
| 2   | Urine          | s. xylosus | +      | +          |
| 3   | Urine          | s. xylosus | +      | +          |
| 4   | Ear swab       | s. xylosus | -      | +          |
| 5   | Urine          | s. lentus  | +      | -          |
| 6   | Urine          | s. lentus  | +      | -          |
| 7   | Ear swab       | s. lentus  | -      | +          |
| 8   | Vaginal swab   | s. lentus  | +      | -          |
| 9   | Wound swab     | s. lentus  | -      | -          |
| 10  | Skin (carrier) | s. lentus  | -      | -          |
| 11  | Skin (carrier) | s. lentus  | +      | -          |
3.2 Susceptibility to antibiotic

Regarding to antibiotics Susceptibility the results showed 20 (65 %) of CONs were resistant to methicillin oxacillin and cefoxitin as shown in figure (3) were agreement with [29]. According to the studied report all isolates (100%) of CONs were resistance to oxacillin [30]. Two basic mechanisms are responsible for resistance of Staphylococci to the β-lactam antimicrobial. First the β-lactamase production that destroys these agents, second alteration of proteins located in the cellular wall of the bacteria called penicillin-binding protein [31].

![Image](image_url)

**Figure 3.** Resistance of CONs to antibiotic

Cefoxitin is considered to be an excellent inducer of mecA gene expression, therefore staphylococci resistance to methecillinoxacillin should be considered resistance to cefoxitin [32].
The similarity between clinical and carrier isolates are expressed by the mecA gene with high percentage in S. lentus and S. xylosus. This finding was very much similar to other studies[33]. The mecA gene which controls the synthesis of an additional penicillin-binding protein PBp in methicillin – resistance staphylococcus [34]. The result of PCR amplification appearance that all isolates (100%) had mecA gene (figure 4).

**Figure 4.** Amplification of mec A gene by PCR

Twenty isolates of CONs carried mecA genes, represented by 13 S. lentus and 7 isolates of S. xylosus, these were in agreement with the results and found that the mecA gene was detected in all isolates of CONs. A good coloration was observed between phenotypic oxacillin susceptibility testing results and PCR amplification results for CONs. These results were in agreement with the study [35], which reported that 95.8 % isolates were PCR positive for mecA.

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