Molecular study of Methicillin Resistant Staphylococcus lugdunensis (MRSL) Isolates in Hilla city, Iraq
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Abstract:

Out of 690 clinical samples collected from different site (wound, burn, blood culture, sub axillary, urine, stool, sputum, throat, ear, skin lesion, high vaginal, and other different swabs), a total of 178 coagulase negative Staphylococci (CoNS) isolates were recovered. Based on phenotypic characteristics, CoNS were identified into 10 different species; 22 isolates were belonged to Staphylococcus lugdunensis. Two specific genes for S. lugdunensis were used (tanA gene and fbl gene) to confirm identification. Both of these specific genes were detected in 15 (68.1 %) of 22 isolates that identified phenotypically. The remaining 7 isolates (31.9 %) were re-identified as S. pseudolugdunensis. β-lactam resistance screening test showed that 11 (73.3 %) of S. lugdunensis isolates were ampicillin resistant. The results of oxacillin screening test and Oxacillin MIC showed that 7 of the 15 (46.6 %) S. lugdunensis isolates were oxacillin resistant; all these were resistant to ampicillin. The antibiotic susceptibility test by Disc Diffusion test and MIC to 16 antibiotics showed that resistance rates towards these antibiotics. Eight of fifteen S. lugdunensis isolates (53.3 %) were β-lactamase producer. All these isolates were Ampicillin resistant.

Results of mecA gene found that mec A gene was detected in 6 (40 %) of 15 S. lugdunensis. All of these 6 isolates (S1, S2, S3, S4, S5, and S6) were resistant to oxacillin. One isolate (S7) was resistant to Oxacillin but mecA was not detected in this isolates. This study is a first record of isolation and characterization of MRSL form clinical samples in Iraq.
Introduction
The coagulase-negative staphylococci (CoNS) are a large group of Gram positive bacteria most often found colonizing the skin and mucosal surfaces of humans and other mammals (1). Several species of CoNS are recognized as potential pathogens, mainly causing nosocomial infections, often involved in infections related to implanted medical devices such as intravenous catheters, prosthetic heart valves, and orthopedic implants. *Staphylococcus lugdunensis* is a coagulase-negative staphylococci first described by Freney and his colleagues in 1988 (2). The organism is found as a skin commensal in healthy individuals. *S. lugdunensis* has been implicated in invasive diseases, especially fulminant native and prosthetic-valve endocarditis (3). Other invasive infections include brain abscess and meningitis, skin abscesses and soft tissue infections, spondylodiscitis, foreign body infections, and peritonitis (4). *S. lugdunensis* shares a number of potential virulence factors with *S. aureus*. In particular, *S. lugdunensis* may express a clumping factor and produce a thermostable DNase (5). *S. lugdunensis* produces a tannase (tannin acyl hydrolase) that degrades hydrolysable tannins (6). The phenotypic biological tests, such as the ornithine decarboxylase test and genotypic molecular tests have been developed to identified this bacteria (7). Several nucleic acid targets that permit the differentiation of *S. lugdunensis* from other CoNS using molecular methods. These include the 16S rRNA gene, which was used to confirmative identity of *S. lugdunensis* isolate (8). The *rpoB* gene also is specific to *S. lugdunensis* (4). The *tana* gene that coded tannase acyle hydrolase were detected in *S. lugdunensis* (6). A fibrinogen-binding protein known as Fbl that encoded by *fbl* gen is specific to *S. lugdunensis* (9). The *meca* gene has been reported in several data, the first in a neonate with *S. lugdunensis* (MRSL) that produces an alternative penicillin binding protein (PB2A) (10). Although several researchers have been reported phenotypic and molecular characterization of Methicillin resistant *S. lugdunensis* (MRSL) worldwide, little or no information are available about these resistant bacteria in Iraq.

Therefore the main goals of this study were to isolate and determine the antibiotic resistance patterns of these important bacteria from clinical samples and detecting the presence of *meca* gene that encodes methicillin resistance to confirm being MRSL.

Materials and methods
Patients and sample collection:
This study included 630 patients (aged 2 days-70 years) suffering from different infections who admitted to four health centers in Al-Hilla city. These patients were admitted to different hospital wards, in addition to swabs taken from private clinics during a period extending from November 2012 to the end of May 2013. Different swabs 690 were generally collected from different site (wound, burn, blood culture, sub axillary, urine, stool, sputum, throat, ear, skin lesion, high vaginal, and other different swabs). Each sample was immediately inoculated on the blood agar plates, and mannitol salt agar. The swab has been inoculated on culture media and incubated aerobically for 24 hours at 37 °C. Information about age, antibiotic usage, residence and hospitalization of patients were taken into consideration.

Bacterial isolates
*Staphylococcus lugdunensis* isolates were recovered and identified based on their morphology, Gram-staining, catalase test, coagulase test, and ornithine decarboxylation test (11). Identification was confirmed by using two specific genes (*tana* and *fbl* genes) by PCR assay (12).

Screening of β-Lactam (Ampicillin and Oxacillin) Resistant Isolates:
Fifteen *S. lugdunensis* isolated were subjected to β-lactam resistance screening test as a phenotypic selection test. Preliminary screening of *S. lugdunensis* isolates resistance to β-lactam antibiotics was carried out by using pick and patch method on Muller-Hinton agar plates supplemented with ampicillin. All of 15 *S. lugdunensis* isolates were subjected to oxacillin resistance screening test by using the same method on Muller-Hinton agar plates supplemented with 4% NaCl and oxacillin 6 μg/ml (13).

Antimicrobial Susceptibility Testing
The antimicrobial susceptibility patterns of isolates to different antibiotics were determined using Disk Diffusion Test and interpreted according to (13). The following antibiotics were obtained (from Oxoid/U.K, Himedia/India) as standard reference disks as known potency for laboratory use: Ampicillin (10μg), Oxacillin (5μg), Cloxacillin (5μg), Cefoxitin (30μg), Amoxicillin-Clavulanate (20/10 μg), Cefixime (30 μg), Ceftriaxone (30 μg), Imipenem (10 μg), Azithromycin (15 μg) Doxycycline (30 μg). The susceptibility to ampicillin, oxacillin and Vancomycin were also determined using two-fold agar dilution method.

Detection of β-lactamase production:
The present study included 15 isolates were tested to detect their ability to produce β- lactamase. Rapid iodometric method was used for detection of β-lactamase production (14).

Detection of tanA, fbl and mecA genes
Three genes were detected in present study, first tanA gene that coded to tannase acyl hydrolase enzyme that degrades tannin. The 2nd gene was fbl gene that coded to fibrinogen binding protein. The third gene that detected in present study was mecA that responsible for Oxacillin/Methicillin resistance by coding to Penicillin binding protein (PBP2a) the primer sequence of these genes were (tanA F: AGCATGGGCAATAACAGCAGTAA , tanA R: GCTGCGCCAATTTGTCTAAATAT) 239bp, the condition were 95ºC 3min 1x, 94ºC 20sec, 60ºC 20sec 25X, 72ºC ,20 sec., 72ºC 5min 1x (12). (fbl F: GTAAATAGCGAGGCACAAGC , fbl R: GTGAAATCGTATCTGCCCCT) 425bp, the condition were 94ºC 3min 1x, 94ºC 1min, 60ºC 1min 30X, 72ºC 1min,72ºC 5min 1x (15). (mecA F: TCCAGGAATGCAGAAAGACCAAAGC , mecA R: GACACGATAGCCATCTCTGTTGG) 499bp, the condition were 94ºC 3min 1x, 94ºC 1.5min, 55ºC 1min 36x, 72ºC 1min, with final step 72ºC 10min 1x (16).

Results and Discussion
Isolation and Identification of Staphylococcus lugdunensis isolates
A total of 690 clinical samples were collected, 602 (87.24%) gave positive growth on blood agar medium, while 88 (12.76%) gave no growth. The reason of negative culture may be attributed to fungal infection, viral infection, or fastidious bacteria that might be lost during transporting or cannot be growing on selective media used in this study.

Out of 393 Gram positive bacteria, 306 (77.8%) were identified as staphylococci based on morphological characteristics and biochemical tests. According to result of coagulase test, the 306 staphylococci isolates were divided into coagulase positive 128 (41.8%) and coagulase negative 178 (58.2%) (Table 1).

<table>
<thead>
<tr>
<th>Source</th>
<th>No.</th>
<th>(%)</th>
<th>CoNS No.</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wound swabs</td>
<td>105</td>
<td>(17.4)</td>
<td>42</td>
<td>(23.56)</td>
</tr>
<tr>
<td>Ear swabs</td>
<td>28</td>
<td>(4.6)</td>
<td>6</td>
<td>(3.37)</td>
</tr>
<tr>
<td>Urine swabs</td>
<td>50</td>
<td>(8.3)</td>
<td>11</td>
<td>(6.17)</td>
</tr>
<tr>
<td>Skin lesion swabs</td>
<td>25</td>
<td>(4.1)</td>
<td>10</td>
<td>(5.6)</td>
</tr>
<tr>
<td>Throat swabs</td>
<td>32</td>
<td>(5.3)</td>
<td>11</td>
<td>(6.17)</td>
</tr>
<tr>
<td>Burn swabs</td>
<td>80</td>
<td>(13.2)</td>
<td>15</td>
<td>(8.4)</td>
</tr>
<tr>
<td>Blood culture</td>
<td>69</td>
<td>(11.4)</td>
<td>13</td>
<td>(7.3)</td>
</tr>
<tr>
<td>Sputum</td>
<td>36</td>
<td>(5.9)</td>
<td>4</td>
<td>(2.24)</td>
</tr>
<tr>
<td>Sub axillary swabs*</td>
<td>64</td>
<td>(10.6)</td>
<td>52</td>
<td>(29.2)</td>
</tr>
<tr>
<td>HVS**</td>
<td>23</td>
<td>(8.3)</td>
<td>2</td>
<td>(1.12)</td>
</tr>
<tr>
<td>Stool swabs</td>
<td>38</td>
<td>(6.3)</td>
<td>2</td>
<td>(1.12)</td>
</tr>
<tr>
<td>other</td>
<td>52</td>
<td>(8.6)</td>
<td>10</td>
<td>(5.6)</td>
</tr>
<tr>
<td>Total</td>
<td>602</td>
<td>(100)</td>
<td>178</td>
<td>(100)</td>
</tr>
</tbody>
</table>
Result of present study was similar to that of Bouza (17), who found that bacterial isolates from clinical samples included 70.7 % Gram positive, 22.2 % of Gram negative, and 7.2% of yeast, they also found that S. aureus and CoNS constituted 40% and 60% respectively. In a local study, Al-Fuadi (18) found that total of 148 bacterial isolates represented by different Gram- positive and Gram-negative bacteria in a percentage of (77%) and (23%) respectively, and they found that a total of 100 Staphylococcus isolates, Only 31 (31%) isolates were belonged to S. aureus. This difference may be belonged to variation of samples collected in this study. Results also showed that the highest percentages of CoNS in Sub axillary swabs and wound swabs were 29.2% and 23.5% respectively. The high frequency of CoNS in these samples might be due to the fact that Staphylococcus species are frequent commensal bacteria on the human skin and mucous surfaces. CoNS were identified depending on phonotypical, biochemical, and physiological tests.

The prevalence of S. lugdunensis was 22 (12.3%), which is higher than results of several researchers. This may be due to the fact that depending on phenotypic characteristics alone is insufficient and may result in misidentification of S. lugdunensis. So, the present study depended (in addition to phenotypic characteristics) on the genotypic characteristics (PCR) to confirm the result. Depending on PCR results, Out of 22 of CoNS that identified phenotypically as S. lugdunensis isolates, 15 (8%) were identified as S. lugdunensis while the other seven isolates were belonged to S. pseudolugdunensis.

Clinical isolates were as follows: Sub auxiliary swab (4) skin swabs (2), wound (3), burn (1), blood (1), throat (2), ear (1), peritonitis (1), while no S. lugdunensis isolates were recovered from urine, sputum swabs, stool swabs, high vaginal swabs. skin swabs represented folliculitis, boils, and abscesses. Researcher (19) found that it is constituted 9 % of CoNS isolates from blood culture, while other researchers found that S. lugdunensis constituted only 3.3 % of CoNS collected from different samples (20).

Molecular Characterization of S. lugdunensis Isolates:
Definite phenotypic identification of a Gram-positive, catalase-positive coccus as S. lugdunensis implies a negative tube coagulase test and positive ornithine decarboxylase activities (21). However, complete hemolytic, yellow pigmentation, and detection of a fibrinogen affinity factor, although not consistently expressed by S. lugdunensis, may lead to its misidentification as S. aureus (1). S. lugdunensis is an unusually virulent coagulase-negative species, associated with severe infection. So, using single-step, speciesspecific PCR protocol for S. lugdunensis identification is very important (15).

Detection of tanA gene of Staphylococcus lugdunensis
The specific tanA gene for S. lugdunensis was detected in 15 (68.1 %) of 22 isolates that identified phenotypically. These 15 isolates were identified as S. lugdunensis (Figure-1). The remaining 7 isolates (31.9 %) were re-identified as Staphylococcus pseudolugdunensis (22). Result also found that S. aureus and S. epidermidis that used as negative control had no tanA gene which confirms the result of Nogochi and his co-worker (2010) who found that no gene or protein homologous to tanA were found in a similarity search using published databases such as Gen Bank. These results strongly suggest that tanA is specific to S. lugdunensis.
Figure (1): Gel electrophoresis of PCR of tanA amplicon (239bp) product: Lane L: Ladder (1000-bp ladder), Lanes (S1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14) No. of S. lugdunensis isolates from different clinical samples.

Detection of fbl gene of Staphylococcus lugdunensis isolates:
A suitable nucleic acid target to diagnosed S. lugdunensis is fbl gene, that encoding a fibrinogen-binding adhesin (15). The gene was detected in all 15 S. lugdunensis isolates that was positive to tanA in this study (Figure -2), while no amplification product was obtained from S. aureus and S. epidermidis isolates that used as negative control as in Figure -3. According to results of PCR, among 22 S. lugdunensis that diagnosed phenotypically, 15 isolates were found to be positive to tanA and fbl genes that were specific to S. lugdunensis (4), so other isolates (No.=7) were diagnosed as S. pseudolugdunensis (22).

Figure (2): Gel electrophoresis of PCR of fbl amplicon (425bp) product: Lane L: Ladder (1000-bp ladder), Lanes (S1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14) No. of S. lugdunensis isolates from different clinical samples.
Figure (3): Gel electrophoresis of PCR of fbl amplicon (425bp) product: Lane L: Ladder (1000-bp ladder), Lanes (S1, and 15) No. of S. lugdunensis isolates. C1: S. aureus, C2: S. epidermidis.

**Primary Screening of β-Lactam (ampicillin and oxacillin) Resistant Isolates:**

The results of screening test showed that 11 isolates (73.3 %) of *S. lugdunensis* were resistant to ampicillin, while 4 (26.7 %) were ampicillin sensitive. All these isolates were able to grow normally in the presence of ampicillin, this may be attributed to most of *S. lugdunensis* isolates (about 90% of them) are coming from several infectious sources (nosocomial infections and other anatomical sites) that its resistant to penicillin due to production of β-lactamases that act in the hydrolysis of β-lactam ring of penicillin which is transformed into acid neutralizing its bactericidal effect (23).

The results of oxacillin resistant screening test showed that 7 of the 11 (63.6 %) β-lactam resistant *S. lugdunensis* isolates were oxacillin resistant. Resistance to oxacillin is due to the fact that *S. lugdunensis* isolates have β-lactamase that reduces efficiency of β-lactam antibiotic. This result was in concordance with study of (24) they were referred to identifying methicillin resistance by oxacillin MIC, 76.5% (13 out of 17) and 47.1% (eight out of 17) of strains were considered resistant by the Vitek 2 system and the Wider system.

**Susceptibility of Staphylococcus lugdunensis to β-Lactam Antibiotics**

The results revealed that 11 of 15 *S. lugdunensis* isolates showed high resistance (73.3 %) to ampicillin (Figure-4). Results also showed that the resistance rate to oxacillin, Cloxacillin, were 46.6 %. Methicillin replaces methicillin as oxacillin is stable under storage conditions, and methicillin actually is an excellent inducer of the *mecA* gene. Ezekiel (25) isolate three strains of *S. lugdunensis* of 149 CoNS, all isolates were resistant to oxacillin and other β- lactam antibiotics. Tan (21) in Singapore found that resistance to oxacillin was detected in 5% of isolates.

Results of cefoxitin (2nd generation), ceftriaxone and cefexime (3rd generation), showed that the percentages of *S. lugdunensis* resistant isolates were substantial to these antibiotics: 46.6%, 53.3%, 40%, respectively (Figure-4). These results can be explained by the fact that all staphylococcal strains produce β-lactamase which destroys the β-lactam ring resulting in inactive products (26). Tan (21) found that resistance to cefoxitin was detected in 5% of isolates. The resistance rates to amoxiclav and ceftazidime-clavulanic acid were 60% and 53.3% respectively. Clavulanic acid can inhibit the action of β-lactamase enzymes that causes decrease in the resistance of bacteria to β-lactam antibiotics (27). Results found that *S.lugdunensis* isolates were susceptible to imipenem (80%). Imipenem inhibits bacterial cell wall synthesis by binding to and inactivating PBPs (28).

**Susceptibility of Staphylococcus lugdunensis to non β-Lactam Antibiotics**

Result of this study regarding susceptibility to amikacin, found that the isolates showed low level of resistance (46.6 %) to this antibiotic. The resistance rate of azithromycin was 53 %, (Figure-4). This resistance may be attributed to the efflux mechanism in staphylococci which is mediated by MsrA; a protein that induced by clarithromycin, azithromycin and telithromycin, and encoded by *msr A*.
gene (29). Result of this study regarding susceptibility to clindamycin, found that the isolates showed low level of resistance (46.6 %) to this antibiotic. Researcher (30) found 10% of isolates were resistant to clindamycin. *Staphylococcus lugdunensis* isolates results showed (73%) resistance to doxycycline. Tan (21) in Singapore found that resistance to tetracycline was 12% of isolates. The percentage of resistance for Trimethoprim-sulfamethoxazole was 66%. Sulfonamides inhibit dihydropteroate synthase, which blocks folate biosynthesis. This, in turn, leads to defective thymidine biosynthesis (31). Results of this study revealed that *S. lugdunensis* isolates showed that the level of resistance to rifampicin (66 %). Rifampin acts by interacting specifically with the β subunit of the bacterial RNA polymerase encoded by the rpoB gene. Rifampin resistance in *Escherichia coli* and *S. aureus* is due to alterations in the target leading to a reduced affinity of the enzyme for the antibiotic (32).

**Results of antibiotic resistance by MIC**

In this study, 11 of 15 (73.3 %) *S. lugdunensis* isolates were resistant to ampicillin (≥ 128μg/ml) while 4 of 15 were having MIC values reached to 2 μg /ml. The MIC values of *S. lugdunensis* isolates against Oxacillin revealed that 5 of 15 isolates reached to 32 μg/ml, while MIC value of 2 isolates was ≥ 64μg /ml. Six Oxacillin resistant isolates (S1, S2, S3, S4, S5, and S6) having *mecA* gene, but one (S7) didn't has such gene. Out of 15 *S. lugdunensis* isolates (detected by MIC method), 14 isolates (93.2 %) were sensitive to Vancomycin, while only one isolate (6.8%) showed reduced susceptibility to vancomycin 8 μg/ml (intermediate resistant). Bourgeois (32) found that 6 of 13 *S. lugdunensis* isolates were tolerant to vancomycin. No isolates showed any degree of resistance to Vancomycin as in many data. The *van* genes that are responsible for resistance (*van* genes) are inducible and transferable and confer high-level resistance to vancomycin (33).

![Figure-4: Percentages of antibiotic resistance among *Staphylococcus lugdunensis* isolate](image-url)
Detection of β-lactamase production:
Eight isolates (53.3 %) were β-lactamase producer. All these isolates were ampicillin resistant, seven of eight β-lactamase producing isolates were oxacillin resistant, while remaining one was oxacillin sensitive. Six of eight were having meca gene (Table -2).
Mateo (24) found that 11.8% of S. lugdunensis were β- lactamase producers. Several authors reported that the percentages of β-lactamase-positive S. lugdunensis were vary from 24 to 40% in U.S isolates collections (34). Papapetropoulos (35), who isolated 14 S. lugdunensis from clinical specimens (abscesses and wound) from (250 beds) in Athens, Greece, 5 (30.2%) of S. lugdunensis were β-lactamase positive. The difference between this study and other studies may be due to the fact that the global using of β-lactam antibiotics in Iraq may which results in induction of bacterial resistance to β-lactams via production of β-lactamase (36).

Molecular detection of MRSL isolates
Detection of meca gene
In this study meca gene was detected in 6 (40 %) of 15 S. lugdunensis (Figure-5). All of these 6 isolates (S1, S2,S3, S4, S5, and S6) were resistant to oxacillin (Table 2). One isolate (S7) was resistant to oxacillin but meca was not detected in this isolates. This resistant may due to mechanism other than changing PBPs (meca) like hyper production of β-lactamase, efflux mechanism in staphylococci which is mediated by MsrA, chemical modification , changing the target of antibiotic and/ or changing permeability of membrane (37).

![Figure (5): Gel electrophoresis of PCR of meca amplicon (499bp) product](image)

Staphylococcus lugdunensis is generally considered to be susceptible to oxacillin. Several studies reported negative PCR results when screening for meca, but among reports in the English literature meca has been detected in two S. lugdunensis isolates (38),(8). (8) reported a case of MRSL causing bloodstream infection in a neonate with an oxacillin MIC˃256 mg/L having meca gene. In 2008, Tan (21) found five (4.7%) S. lugdunensis strains carrying the meca gene in a collection of 106 clinical isolates.

Table 2: relationship between ampicillin, oxacillin resistant with present of meca gene and β-lactamase production in Staphylococcus lugdunensis isolated:
<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Ampicillin resistant</th>
<th>Oxacillin resistant</th>
<th>mecA</th>
<th>β-lactamase production</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S2</td>
<td>+</td>
<td>+</td>
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<td>S15</td>
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</tbody>
</table>

Conclusion
The present study can conclude the following points:

1. The ratio of coagulase negative staphylococci was higher than coagulase positive staphylococci.
2. A highest percentages of *S. lugdunensis* isolate were recovered from sub axillary, wound swabs, and skin swabs, samples, so the results from this study reinforce the propensity of *S. lugdunensis* to be associated with acute cutaneous infections.
3. Although many other reports stated that *mecA* gene presents in low percentage in *S. lugdunensis*, however, in present study, high rate of *mecA* is present in these bacteria.

References: