Mannitol Fermenting Methicillin-Resistant Coagulase Negative Staphylococci Isolated From Diabetic Foot Infections

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ABSTRACT

Detection of mannitol fermenting coagulase-negative staphylococci is frequently unnoticed when *Staphylococcus aureus* is screening in the laboratory. On the other hand, the emergence of coagulase-negative staphylococci as critical human pathogens need dependable methods for the identification of clinically significant coagulase-negative staphylococci to understand the epidemiology of infections caused by these bacteria. The study aimed to identify mannitol fermenting coagulase-negative staphylococci that assumed to be *Staphylococcus aureus* as they formed yellow colonies on Mannitol Salt agar plates. Samples were taken from eighty-four patients with diabetic foot infections. The specimen was cultured on Blood agar and Mannitol Salt agar. Mannitol fermenting coagulase-negative staphylococci isolates diagnosed through Vitek2 system then confirmed by detecting 16S rRNA gene and absence of the nuc gene. Antibiotic sensitivity and methicillin resistance were detected by Vitek2 system, then methicillin resistance was confirmed by Oxacillin Salt Agar Screen test and detection of the mecA gene. Out of 81 *Staphylococcus* isolated from foot and nose of diabetic foot patients, twenty isolates were mannitol fermenting coagulase-negative staphylococci, they related to following species; *Staphylococcus haemolyticus, staphylococcus lentus, Staphylococcus xylosus, Staphylococcus lugdunensis, Staphylococcus hominis, Staphylococcus galinarum* and *Staphylococcus saprophyticus*. The majority of them (85%) were phenotypically methicillin-resistant and genotypically harbouring mecA gene. 80% were resistant to Erythromycin, 70% to Clindamycin, 35% to Trimethoprim-Sulphamethoxazole, 30% to Gentamicin and Rifampicin, 15% to Levofoxacin and Teicoplanin. 30% expressed inducible clindamycin resistance.

INTRODUCTION

*Staphylococcus aureus* (*S. aureus*) is the primary etiological agent of skin and nares, (Ayeni et al., 2017; Anyanwu, 2013), and most common causative agent in diabetic foot infections (Viswanathan et al., 2019). Due to its clinical importance, a large number of diagnostic tests are used to identify *S. aureus*; growth on Mannitol Salt Agar plate (MSA) is the most widely used. MSA plate was developed in the year 1945 to selectively isolate pathogenic staphylococci in one step (Thakur et al., 2017). MSA is a selective medium containing 7–9 % NaCl.
which allows *S. aureus* to grow and produce yellow colonies with yellow zones due to fermentation of mannitol sugar which in turn lead to drop in the medium’s pH. In contrast, coagulase-negative staphylococci (CONS) produce pink to red colonies and no change in the medium colour (*Ayeni et al., 2017; Ayeni and Odumosu, 2016*).

There are some reports about CONS that they also can ferment mannitol and produce yellow colonies on MSA (*Ayeni, 2018; Shittu et al., 2006*). Mannitol Salt positive CONS interrupt the isolation and then identification of *S. aureus* on the primary plate. Unfortunately, false-positive results caused by coagulase-negative staphylococci can lead to overestimation of *S. aureus* infection rates (*Sirobhushanam et al., 2019*).

Coagulase-negative staphylococci considered as one of the most frequently isolated pathogens in a clinical laboratory. They are now gradually becoming an essential causative agent in various infections like wounds (*Shittu et al., 2006; Nagaraju-vanaparti, 2019*). They are increasingly recognised as pathogens in case of diabetic foot wound infections (*Patil and Mane, 2017*). CONS acquired multiple antibiotic-resistance mechanisms, especially methicillin resistance (*Katragadda and Venkateswaran, 2018*). The problem is worsened by the fact that methicillin-resistant staphylococci, in addition to beta-lactam antibiotics, are resistant to other antibiotic classes including macrolides, aminoglycosides, fluoroquinolones and tetracyclines (*Ugwu et al., 2015*). There is a believed that CONS might act as a significant reservoir for antibiotic resistance genes, which might be transferred among *Staphylococcus* genus (*Adekanmbi et al., 2019; Salimi et al., 2016*). Therefore, accurate identification of CONS species is of diagnostic value and clinical importance.

This study aimed to identify these CONS that isolated from diabetic foot infections and nares of diabetic patients, which assumed to be *S. aureus* as they form yellow colonies on MSA plates.

**MATERIALS AND METHODS**

**Study place and period**

From December 2016 to January 2018 eighty-four patients with diabetic foot ulcer who hospitalised in Rizgari and Hawler teaching hospitals in Erbil city/ Iraq were included in this study.

**Ethical approval**

The ethics committee of the College of Medicine/Hawler Medical University approved this study before conducting the study (paper code: 15- date: 23/4/2016). Informed consent was collected from patients before obtaining Specimens.

**Inclusion criteria**

The patient had diabetes mellitus with foot ulcers, accidentally diagnosed as diabetic Mellitus after admission with a foot ulcer, patient with gangrene of the foot complicated by diabetes.

**Exclusion criteria**

Patient not willing to participate, had foot infections but were not diabetic, had gangrene of the foot. Still, the aetiology wasn’t due to diabetes complication, had a healed ulcer site, pregnant or under 18 years old.

**Collection and culture of specimens**

Specimens were collected aseptically from infected foot lesions and nose of diabetic patients by using a sterile cotton swab (*Lipsky et al., 2016*) and cultured on Blood agar and Mannitol Salt agar plates (Lab M / UK), then incubated at 37°C and checked after 24 - 48 hours.

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**Figure 1: Mannitol fermenting CON Staphylococcus haemolyticus on Mannitol Salt agar media**

**Presumptive identification of bacteria**

After growth, if bacteria in the culture media were a mix, they purified by streak plate method to create a pure culture of one bacteria and determine the phenotypic character of the bacteria like mannitol fermentation and yellow pigment production.

**Identification by Vitek2 system**

The Vitek2 system (BioMérieux Vitek, France) was used in this study to confirm the identification of all isolated bacteria.

**Antibiotic susceptibility testing by Vitek2 system**

Susceptibility test card “AST-GP580 TEST KIT-REF22233” for Gram-positive was used to perform
Table 1: Primers to detect CONS of bacteria identity genes and methicillin-resistant genes

| Gene name | Nucleotide sequence | Bp | References |
|-----------|---------------------|----|------------|
| 16S rRNA-F | 5’-GTA GGT GGG AAG CGT TAT CC - 3’ | 218 | (Manal et al., 2009) |
| 16S rRNA-R | 5’-CGC ACA TCA GGG TCA G - 3’ |  |  |
| nuc-F | 5’-GCG ATT GAT GGT GAT ACG GTT - 3’ | 280 | (Manal et al., 2009) |
| nuc-R | 5’-AGC CAA GCC TTG ACG AAC TAA AGC - 3’ |  |  |
| mecA-F | 5’-CTC AGG TAC TGC TAT CCA CC - 3’ | 448 | (Taha et al., 2017) |
| mecA-R | 5’-CAC TTG GAT TAT CTT CAC C - 3’ |  |  |

Table 2: Mannitol-fermenting CONS identity confirmed by Vitek2 system

| No | Bacteria | No. of isolates |
|----|----------|-----------------|
| 1  | *Staphylococcus haemolyticus* | 7 |
| 2  | *Staphylococcus lentus* | 6 |
| 3  | *Staphylococcus xylosus* | 2 |
| 4  | *Staphylococcus lugdunensis* | 2 |
| 5  | *Staphylococcus hominis* | 1 |
| 6  | *Staphylococcus galinarum* | 1 |
| 7  | *Staphylococcus saprophyticus* | 1 |

Table 3: Mannitol-fermenting CONS identity confirmed by 16S rRNA and nuc genes

| No. | Patient code | CONS of bacteria | Location of isolation | 16S rRNA gene | Nuc gene |
|-----|-------------|------------------|-----------------------|---------------|---------|
| 1   | 5           | *S. haemolyticus*| Nose                  | Positive      | Negative |
| 2   | 25          | *S. haemolyticus*| Left Foot             | Positive      | Negative |
| 3   | 34          | *S. haemolyticus*| Nose                  | Positive      | Negative |
| 4   | 63          | *S. haemolyticus*| Nose                  | Positive      | Negative |
| 5   | 71          | *S. haemolyticus*| Nose                  | Positive      | Negative |
| 6   | 71          | *S. haemolyticus*| Right Foot            | Positive      | Negative |
| 7   | 84          | *S. haemolyticus*| Left Foot             | Positive      | Negative |
| 8   | 19          | *S. lentus*      | Nose                  | Positive      | Negative |
| 9   | 34          | *S. lentus*      | Right foot            | Positive      | Negative |
| 10  | 42          | *S. lentus*      | Left Foot             | Positive      | Negative |
| 11  | 43          | *S. lentus*      | Left Foot             | Positive      | Negative |
| 12  | 73          | *S. lentus*      | Nose                  | Positive      | Negative |
| 13  | 73          | *S. lentus*      | Left Foot             | Positive      | Negative |
| 14  | 6           | *S. xylosus*     | Right Foot            | Positive      | Negative |
| 15  | 55          | *S. xylosus*     | Left Foot             | Positive      | Negative |
| 16  | 47          | *S. lugdunensis* | Nose                  | Positive      | Negative |
| 17  | 50          | *S. lugdunensis* | Nose                  | Positive      | Negative |
| 18  | 14          | *S. hominis*     | Left Foot             | Positive      | Negative |
| 19  | 77          | *S. galinarum*   | Left Foot             | Positive      | Negative |
| 20  | 84          | *S. saprophyticus*| Right Foot           | Positive      | Negative |
Table 4: Confirmation of mannitol fermenting CONS resistance to methicillin by phenotype and genotype methods

| No. | Patient code | Bacteria      | Vitek2 system | Oxacillin Salt-Agar Screen Test | mecA gene |
|-----|--------------|---------------|---------------|---------------------------------|-----------|
| 1   | 5            | S. haemolyticus | Positive      | Negative                        | Positive  |
| 2   | 25           | S. haemolyticus | Positive      | Positive                        | Positive  |
| 3   | 34           | S. haemolyticus | Positive      | Positive                        | Positive  |
| 4   | 63           | S. haemolyticus | Positive      | Positive                        | Positive  |
| 5   | 71           | S. haemolyticus | Positive      | Positive                        | Positive  |
| 6   | 71           | S. haemolyticus | Positive      | Positive                        | Positive  |
| 7   | 84           | S. haemolyticus | Positive      | Positive                        | Positive  |
| 8   | 19           | S. lentus      | Positive      | Positive                        | Positive  |
| 9   | 34           | S. lentus      | Positive      | Positive                        | Positive  |
| 10  | 42           | S. lentus      | Positive      | Positive                        | Positive  |
| 11  | 43           | S. lentus      | Negative      | Positive                        | Positive  |
| 12  | 73           | S. lentus      | Positive      | Positive                        | Positive  |
| 13  | 73           | S. lentus      | Positive      | Positive                        | Positive  |
| 14  | 6            | S. xylosus     | Negative      | Negative                        | Positive  |
| 15  | 55           | S. xylosus     | Negative      | Negative                        | Positive  |
| 16  | 47           | S. lugdunensis | Positive      | Positive                        | Positive  |
| 17  | 50           | S. lugdunensis | Positive      | Positive                        | Positive  |
| 18  | 14           | S. hominis     | Positive      | Positive                        | Positive  |
| 19  | 77           | S. galinarum   | No*           | Positive                        | Positive  |
| 20  | 84           | S. saprophyticus| No*          | Positive                        | Positive  |

No* = No Vitek data are given

Methicillin resistance detection in isolated bacteria

1. Vitek2 system
Detection of Cefoxitin and Oxacillin resistance investigated with the same card used in the antibiotic susceptibility testing, as mentioned above.

2. Oxacillin Salt Agar Screen Test
Mueller-Hinton agar (Lab M / UK) plates containing 4% NaCl and six μg/ml of Oxacillin powder were prepared and inoculated with 1 μl of 0.5 McFarland suspension of the bacterium by streaking in one quadrant and incubated at 33 to 35°C for 24 hrs. (because incubating above 35°C may not detect methicillin-resistant strains). Plates were scrutinised with transmitted light for > 1 colony or light film of growth (> 1 colony = oxacillin resistant) (CLSI, 2019).

Extraction of genomic DNA
Genomic DNA extracted by using EzWay™ Genomic DNA Kit, Bacterial (KOMABIOTECH/South Korea) following the manufacturer’s instructions.

Genotype identification of *Staphylococcus* isolates and detection of mecA gene
Primers for the 16S rRNA gene was used to confirm genus, and species-specific nuc gene to confirm *aureus* species (Shittu et al., 2006). Also, methicillin resistance was confirmed by using a primer for detection of mecA gene as illustrated in Table 1

Data analysis
Data were analysed using SPSS version 23.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS
The samples were taken from eighty-four diabetic foot patients who participate in this study. Mean age of the patients was 57.21±11.64 years. Males represented 54.8%, while females were 45.2%.

Out of 81 staphylococci strains, 20 coagulase-negative staphylococci were able to ferment mannitol sugar on Mannitol Salt agar plates, as shown in Table 2 and Figure 1. The Vitek2 system determined the identity of these isolates. Confirmation
made through detecting 16S rRNA gene to confirm them as Staphylococcus genus and through searching for nuc gene (absence of nuc gene confirm phenotypic identification of these isolates), all isolates were positive for 16S rRNA and negative for nuc gene as shown in Table 3.

Majority of these bacteria were resistant to methicillin phenotypically by Vitek2 system method (83.3%) and Oxacillin Salt Agar Screen Test (85%). All of them (100%) found to harbour mecA gene as seen in Table 4

Sixteen (80%) out of 20 Mannitol fermenting CONS were resistant to Erythromycin, 14 (70%) to Clindamycin, 7 (35%) to Trimethoprim-Sulphamethoxazole, while the resistance to Gentamicin and Rifampicin was 6(30%), to Levofloxacin and Teicoplanin was 3(15%). Six (30%) of the isolates expressed inducible clindamycin resistance. None of the isolates was resistant to Moxifloxacin, Linezolid, Vancomycin and Tigecycline. 85% of the isolates were multidrug-resistant (MDR) as they showed resistance to at least three classes of antibiotics Table 5

DISCUSSION

Some microbiologists often depend on differential test and growth on selective media to diagnose bacteria, which may lead to wrong identification of some bacteria and result in the wrong prescription of antibiotics in which may later lead to therapeutic failure. Production of yellow colonies due to fermentation of Mannitol sugar is considered as a presumptive tool to identify S. aureus and to differentiate it from coagulase-negative staphylococci. Still, some researchers reported that some of the CONS also could produce yellow colonies on MSA (Ugwu et al., 2015; Shittu et al., 2006). Repeatedly isolation of CONS from patients with infection should be taken seriously as important human pathogens, dependable methods for proper diagnosis and treatment of CONS are such essential to understand the epidemiology of infections caused by these bacteria (Vanarthi et al., 2017).

Contrary to reports that mentioned MSA as a useful tool in which it can be used to discriminate between S. aureus and CONS. This study provides the first report on different species of CONS that can ferment mannitol as S. haemolyticus, S. lentus, S. xylosus, S. lugdunensis, S. hominis, S. galinarum and S. saprophyticus that isolated from diabetic foot.
infections and grown on MSA with characteristic production of yellow colonies. The Vitek2 system confirmed them, 16S rRNA gene and nuc gene. The absence of the S. aureus species-specific nuc gene confirmed phenotypic identification as CONS (Shittu et al., 2006). (Spanu et al., 2003) misreported the accuracy of Vitek2 system identifying CONS isolates. (Sirobhushanam et al., 2019) reported 7% false-positive results and 31% false-negative results when colonies are identified by colour alone on differential culture media. These isolates were also confirmed phenotypically and genotypically as methicillin-resistant. Same results were published by (Ugwu et al., 2015; Thakur et al., 2017; Shittu et al., 2006). (Sitthisak et al., 2019) mentioned that methicillin-resistant CONS are the predominant cause of nosocomial infections, which greatly limit therapeutic options for opportunistic infections and may play a role in spreading resistances within the community (Falomir et al., 2019; Kumar et al., 2018).

Also (Mergenhagen et al., 2020) suggests that a negative methicillin-resistant nares swab is useful to predict the absence of methicillin resistance in a subsequent culture from a diabetic foot infection because guidelines recommend empiric methicillin resistance coverage in patients who have had methicillin resistance previously. There is toxicity related to antibiotics for empiric methicillin resistance coverage, especially vancomycin, and for that reason they recommend methicillin resistance Staphylococcus aureus nares screening at admission to hospital. Besides resistance to beta-lactam antibiotics, the methicillin-resistant CONS in this study also showed resistance to other antibiotic classes like macrolides, lincosamides, sulfonamides, aminoglycosides and rifamycins. murugesan et al. reported a similar finding. For CONS isolated from nares of hemodialysis patients (Murugesan et al., 2019). Although the degree of importance of multidrug-resistant methicillin-resistant CONS is not clearly understood, they may serve as a reservoir for resistance genes. They may spread from them to pathogenic bacteria within and across species and genera (Ugwu et al., 2015).

This finding indicates that a single phenotypic test cannot provide dependable results in the identification and differentiation of S. aureus with CONS, and a combination of tests should be used to avoid misidentification of isolates.

CONCLUSIONS

This study, along with earlier investigations, indicates that there is the high false identification of S. aureus and other tests are needed to differentiate between S. aureus and CONS colonies that ferment mannitol on MSA. So educating microbiologist working in clinical laboratories about this misidentification and recommending using more than one confirmatory test is so important to decrease the rate of false-positive identification.

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Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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