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

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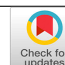
ABSTRACT



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 Levofloxacin and Teicoplanin. 30% expressed inducible clindamycin resistance.

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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; Nagarajuvanaparti, 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.

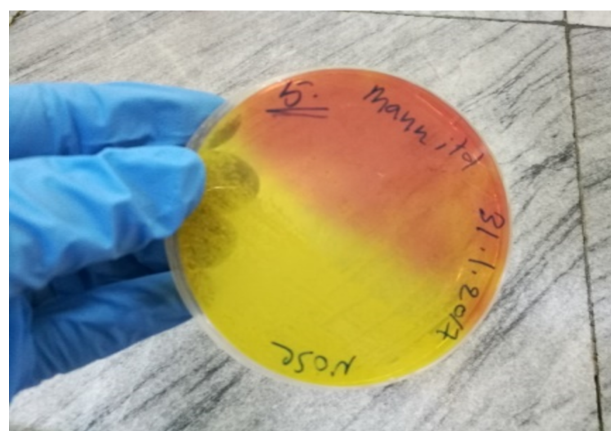

Figure 1: Mannitol fermenting CON Staphylococcus haemolyticus on Mannitol Salt agar media

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-

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Table 1: Primers to detect CONS of bacteria identity genes and methicillin-resistant genes

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

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	Methicillin resistance test results		
			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

REF.22233" for Gram-positive was used to perform susceptibility testing for different antibiotics according to the manufacturer's instructions.

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^{TM} Genomic DNA Kit, Bacterial (KOMABIOTECH/South Korea) following the manufacturer's instructions.

Genotype identification of *Staphylococci* 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 deter-

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Table 5: Antibiotic resistance patterns of mannitol fermenting CONS

No.	Patient code	CONS of bacteria	Resistance to antibiotics
1	5	*S. haemolyticus*	Lev, Ery, Tet, TMP-SMX
2	25	*S. haemolyticus*	Ery, Clin, Tet,
3	34	*S. haemolyticus*	Teic, Tet, TMP-SMX
4	63	*S. haemolyticus*	No resistance to any tested antibiotics
5	71	*S. haemolyticus*	Gen, Ery, Clin, Rif
6	71	*S. haemolyticus*	Gen, Ery, Clin, Rif
7	84	*S. haemolyticus*	Lev, Ery, Tet, TMP-SMX
8	19	*S. lentus*	Gen, Tob, Ery, Clin, Tet, Rif
9	34	*S. lentus*	Gen, Tob, Lev, Ery, Clin, Teic, Rif, TMP-SMX
10	42	*S. lentus*	Ery, Clin, Tet, Rif
11	43	*S. lentus*	Ery, Clin., Tet
12	73	*S. lentus*	Ery, Clin, Tet
13	73	*S. lentus*	Ery, Clin, Tet
14	6	*S. xylosus*	Ery, Clin, Tet, TMP-SMX
15	55	*S. xylosus*	Clin, Tet
16	47	*S. lugdunensis*	Ery, Clin, Tet, Rif
17	50	*S. lugdunensis*	Ery, Clin, Tet
18	14	*S. hominis*	Gen, Tob, Ery, Clin, Teic, Tet, TMP-SMX
19	77	*S. galinarum*	No Vitek data
20	84	*S. saprophyticus*	Gen, Ery, Tet, TMP-SMX

Lev=Levofloxacin,Ery=Erythromycin, Tet=tetracycline, TMP-SMX= Trimethoprim/Sulfamethoxazole, Clin=Clindamycin, Teic=Teicoplanine, Gen=Gentamicin,Rif=Rifampicin, Tob=Tobramycin.

mined 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 (Vanaparthi *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, *16SrRNA* 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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