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Microbiological profile of diabetic foot infections and the detection of mecA gene in predominant *Staphylococcus aureus*

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ABSTRACT

BACKGROUND

Diabetes mellitus (DM) is a serious health problem that is rapidly expanding worldwide. *Staphylococcus aureus* is a pathogenic bacterium which has a number of drug resistant strains. Different variants of this pathogen have been isolated from patients with diabetic foot ulcers - in persons having uncontrolled blood sugar level - all over the world, resulting in high rates of morbidity and mortality. The objective of this study was to determine the prevalence of drug resistant *Staphylococcus aureus* in diabetic foot infections (DFIs).

METHODS

An epidemiological survey was conducted and 300 pus samples were collected from wounds, abscesses, skin and soft tissue lesions of patients having type II diabetes with foot ulcer infections at a tertiary care hospital. Further, the antibacterial susceptibility patterns of all the isolated *Staphylococcus aureus* were determined against methicillin, oxacillin, vancomycin and novobiocin.

RESULTS

Pathogenic bacterial species including coagulase positive and coagulase negative *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Pseudomonas* sp. and *Citrobacter* sp. were identified, among which *Staphylococcus* was the main genus identified. A total of 13 (4.3%) isolates of coagulase positive *Staphylococcus aureus* were resistant to methicillin. Using PCR, 7 (53.8%) staphylococcal isolates were detected with the *mec*A gene.

CONCLUSION

Staphylococcus aureus is the most common cause of DFIs. This study demonstrates that about 53.8% of all methicillin resistant *Staphylococcus aureus* isolates have *mec*A genes. Such a finding is the primary step in understanding and tackling the resistance mechanism.

Keywords: Diabetic foot ulcer, *Staphylococcus aureus*, *mec*A, methicillin

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INTRODUCTION

Diabetes mellitus (DM) which is encountered when the blood glucose level is increased, poses a serious threat to the public health with high rates of morbidity and mortality worldwide.[1] Unfortunately, it affects 463 million adults globally, indicating that DM is the seventh major cause of death [2,3] and this estimation is likely to be increased 1.5-fold in 2045.[4] Diabetes mellitus introduces many complications such as diabetic foot ulcers (DFU), pressure ulcers and other types of venous leg ulcers. Among them, DFU is affecting 15-25% of the diabetic population every year across the world,[5,6] which increases the number of limb amputations by 5- 24% within a period of 6–18 months after the first evaluation. by infecting soft tissues and bony structures.[7-9] The pathogenesis of DFU is unknown [10] and its incidence and severity are based on the effect of host-associated conditions such as neuropathy and also pathogen-linked factors such as colonization, microbial resistance and virulence factors.[11] Initially, DFU would be a single microbial infection, but later it may turn into polymicrobial infections with both aerobic and anaerobic microbes.[12,13] Bacterial virulence factors and the host resistance level play a major role in the diagnosis and management of DFU infections.[14] The treatment of DFU remains a changing one because of the overuse of antibiotics as the clinical symptoms of DFU mimic the ulcer infections. Unfortunately, this phenomenon has an impact on the ecology of the human microbiome, resulting in the occurrence of drug-resistant organisms. Many reports indicate that DFU is caused by a variety of drug-resistant organisms including methicillin-resistant *Staphylococcus aureus* (MRSA) [15,16] and hence the incidences are higher.

Among the microorganisms, *Staphylococcus aureus* is one of the most frequently isolated DFU bacterium which is a commensal as well as human pathogen [17,18] causing approximately 30% of human infections such as sepsis, bacteraemia, pneumonia and skin infections.[19] Methicillin-resistant *Staphylococcus aureus* has been acquired through a variety of risk factors such as prior amputation, previous hospitalization, prior antibiotic usage and stay in chronic care facilities.[20,21] Methicillin-resistant *Staphylococcus aureus* has the ability to resist all the currently used antibiotics which makes the treatment procedures costly, while the side effects related to antibiotics create difficulty in the management of DFU infections.[22] Moreover, *Staphylococcus aureus* genomes consist of variety of genes which are responsible for antibiotic resistance and other virulence factors.

A study on diabetic foot infections showed that polymicrobial cultures were obtained from 83.7% of patients with a rate of isolation of 3.0 ± 1.4 bacteria per patient.[23] A meta-analysis clearly identified a high prevalence of bacterial species/ genera classically associated with diabetic foot infection, e.g. *S. aureus*.[24] The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) identified by this meta-analysis (18.0%) matches closely that of a previous meta-analysis.[25] Studies indicate that the within-country and between-country prevalence of MRSA are heterogeneous.[26,27] In addition, there is a need for evidence-based guidance to prescribe an appropriate drug of choice to concerned patients based on local data. Hence, this study aimed to isolate *Staphylococcus aureus* strains and antibiotic sensitivity profiles from diabetic foot ulcer infections and to investigate for the presence of the *mecA* gene that codes for methicillin resistance in *Staphylococcus aureus*.

METHODS

Research design

An epidemiological study was conducted in both male and female in-patients from the wards of the Government District Hospital, Erode, India, in the period of 07-06-2021 to 23-12-2021.

Sample collection and isolation and identification of *S. aureus* stains

A total of 300 pus samples were collected using sterile swabs from wounds, abscesses, skin and soft tissue lesions of patients having type II diabetes with foot ulcer infections. The swabs were transported to the laboratory without any further delay. The samples were swabbed on to MacConkey, blood and mannitol salt agar plates and incubated overnight at 37° C. Then, the plates were observed for colony formation. The isolated colonies were used for morphological, Gram stain, biochemical analyses (coagulase, catalase, oxidase, indole, methyl red, Voges-Proskauer, citrate utilization, triple sugar iron agar, urease, DNase and gelatinase tests) and carbohydrate fermentation tests (glucose, sucrose, mannitol and lactose).[28]

Determination of antibacterial activity

The isolated and identified *S. aureus* strains were diluted and adjusted to form cell suspensions of 0.5 McFarland units. These suspensions were used for the disc diffusion method as described by Gowri et al.[29] Mueller Hinton agar (MHA) plates were used to study the antibacterial activity of isolated *S. aureus* strains against commercially available antibiotics such as methicillin (5 µg), oxacillin (1 µg), vancomycin (30 µg) and novobiocin (30 µg), which were purchased from Hi Media (India). Briefly, the prepared MHA plate surfaces were swabbed with diluted inocula of *S. aureus* and left for five min. After that, the antibiotic discs were placed individually and the plates were incubated for 24 hrs at 37° C. After incubation, the plates were observed for zones of inhibition.

Detection of m*ec*A from *S. aureus* by polymerase chain reaction

The presence of *mec*A from all isolates of *S. aureus* was determined as described by Akhi et al.[28] using the forward (F5'-CTCAGGTACTGCTATACCACC-3') and reverse (R 5'-CACTTGGTATATCTTCACC-3) primers. Briefly, a single bacterial colony was obtained from a fresh subculture and re-suspended in 100 µl of sterile water and 1 ml of suspension was added to each PCR ready mix. The PCR was programmed as follows: bacterial lysis and DNA denaturation step of 5 min at 95° C; 30 cycles with a 30-s denaturation step at 94° C; a 30-s annealing step at 42° C; a 30-s extension at 72° C; and final 10-min extension step at 72° C. After 30 cycles, the final PCR product was detected by gel electrophoresis.

Gel electrophoresis

For the gel electrophoresis, the resulting product was loaded onto the 1.5 % agarose gel with ethidium bromide along with standard DNA 100 bp marker and the electrophoresis was performed at 50 volts using 1 x Tris-Borate EDTA (TBE) as the running buffer. Then the electrophoresis was stopped by turning off the power supply when the product had migrated a distance sufficient for separation of the DNA fragments. Next, the gel was observed for bands on an UV trans-illuminator.

Statistical analysis

The microbiological experiments were performed in triplicate and all the data were statistically analysed by using IBM SPSS software, version 22.0 (Armonk, NY, USA) and qualitative variables were expressed as percentages.

Ethical clearance

This study was approved by the Departmental Ethical Committee (KSRCAS/DECIII-2021/03), at the Postgraduate and Research Department of Microbiology, K.S. Rangasamy College of Arts and Sciences, Tiruchengode, India and written informed consent was obtained from every participating patient.

RESULTS

Isolation and identification

Out of 300 samples collected from patients having type II diabetes with foot ulcer infections,

Table 1. Biochemical analysis of bacteria isolated from DFU infections

Name of the biochemical tests	*S. aureus*	*E. coli*	*Klebsiella* sp.	*Proteus* sp.	*Pseudomonas* sp.	*Citrobacter* sp.
Gram Stain	+ve cocci	-ve rod	-ve rod	-ve rod	-ve rod	-ve rod
Coagulase	+	-	-	-	-	-
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	-	+	-
Indole	-	+	-	-	-	-
Methyl red	+	+	-	-	-	+
Voges- Proskauer	+	-	+	-	-	-
Citrate utilization	+	-	+	+	+	+
Triple sugar iron agar	K/A, Gas +ve, no H_2S	A/A Gas +ve, no H_2S	A/A, No Gas, No H_2S	K/A Gas +ve, H_2S +ve	K/K No Gas, No H_2S	A/A Gas +ve, H_2S +ve
Urease,	+	-	+	+	-	V
DNase	+	-	-	+	-	-
Gelatinase	+	-	-	+	+	-
Glucose	+	+	+	+	+	
Sucrose	+	+	+	-	-	+
Mannitol	+	+	+	-	+	+
Lactose	+	+	+	-	-	+
Xylose	-	+	+	+	-	+

Notes: + indicates positive, - indicates negative, K/K- Alkali slant and butt, A/A- acid slant and butt, K/A- Alkali slant and acid butt and V-Variable

172 samples were from males (58%) and 128 from females (42%) of the age groups between 45 and 85 years. From the samples, 300 distinct colony morphologies were observed on MacConkey, blood and mannitol salt agar plates and the individual colonies were subjected to Gram's staining reaction, showing that 104 isolates were Gram positive (34.4%) and 196 were Gram negative (65.3%). The results of various biochemical analyses such as coagulase, catalase, oxidase, indole, methyl red, Voges-Proskauer, citrate utilization, triple sugar iron agar, urease, DNase and gelatinase tests, and carbohydrate fermentation tests (glucose, sucrose, mannitol and lactose) to which the isolates were subjected are mentioned in Table 1. Based on the microscopy, biochemical reactions and cultural characteristics, the isolates were identified as *S. aureus*, *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Pseudomonas* sp. and *Citrobacter* sp. and the results are displayed in Table 2. The results also showed that, among 104 Gram positive isolates, 31 were coagulase positive *S. aureus* (10.3 %) and 73 were coagulase negative *S. aureus* (24.3%). In the remaining isolates, 30 were *E. coli* (10.0%), 66 isolates were *Proteus* sp. (22.0%), 45 isolates were *Pseudomonas* sp. (15.0%), 15 isolates *Klebsiella* sp. (5.0%) and 40 isolates were *Citrobacter* sp. (13.3%). The coagulate positive and coagulase negative *S. aureus* isolates were used for further analyses.

Table 2. Distribution of the isolates from diabetic patients with foot ulcers (n=300)

Isolated microbes	n (%)
Coagulase positive *S. aureus* (CPS)	31 (10.3)
Coagulase negative *S. aureus* (CNS)	73 (24.3)
E. coli (EC)	30 (10.0)
Proteus sp. (PR)	66 (22.0)
Pseudomonas sp. (PS)	45 (15.0)
Klebsiella sp. (KL)	15 (5.0)
Citrobacter sp.	40 (13.3)

Table 3. Antibiotics sensitive pattern of S. aureus isolates

S. aureus strains	Antibiotics sensitivity profile[$]								
	Methicillin		Oxacillin		Vancomycin			Novobiocin	
	R	S	R	S	R	S	I	R	S
Coagulase positive (n-31)	13 (41.9)	18 (58.1)	2 (6.4)	29 (93.6)	9 (29.0)	21 (67.7)	1 (3.3)	0 (0.0)	31 (100.0)
Coagulase negative (n=73)	0 (0.0)	73 (100.0)	28 (38.3)	45 (61.7)	22 (30.1)	51 (69.9)	0 (0.0)	17 (23.3)	56 (76.7)

Note: R=Resistant, S=Sensitive, I=Intermediate; data presented as n (%)

Antibiotic sensitivity patterns

The antibiotic sensitivity patterns of all isolated coagulase-positive and coagulase-negative *S. aureus* were determined against above mentioned commercially available antibiotics. The results showed that, among the 31 coagulase positive *S. aureus,* 13 (41.9%) isolates were resistant and 18 (58.1%) isolates were susceptible to methicillin. Two (6.4 %) of the isolates were resistant to oxacillin whereas 29 (93.6%) of the isolates showed susceptibility to the same antibiotic. To the antibiotic vancomycin, 9 (29.0%) isolates showed resistance, 1 (3.2%) isolate was of intermediate resistance and 21 (67.8%) isolates showed susceptibility. All 31 coagulase-positive *S. aureus* were susceptible to novobiocin and at the same time, all 73 coagulase-negative *S. aureus* were susceptible to methicillin (Table 3).

Detection of m*ec*A from *S. aureus*

The presence of *mecA* in the isolated *S. aureus* was detected using the PCR technique and the result is presented in Figure 1. Among the 13 isolates which were susceptible to methicillin, 7 (53.8%) were shown to have the presence of the *mecA* gene after PCR amplification.

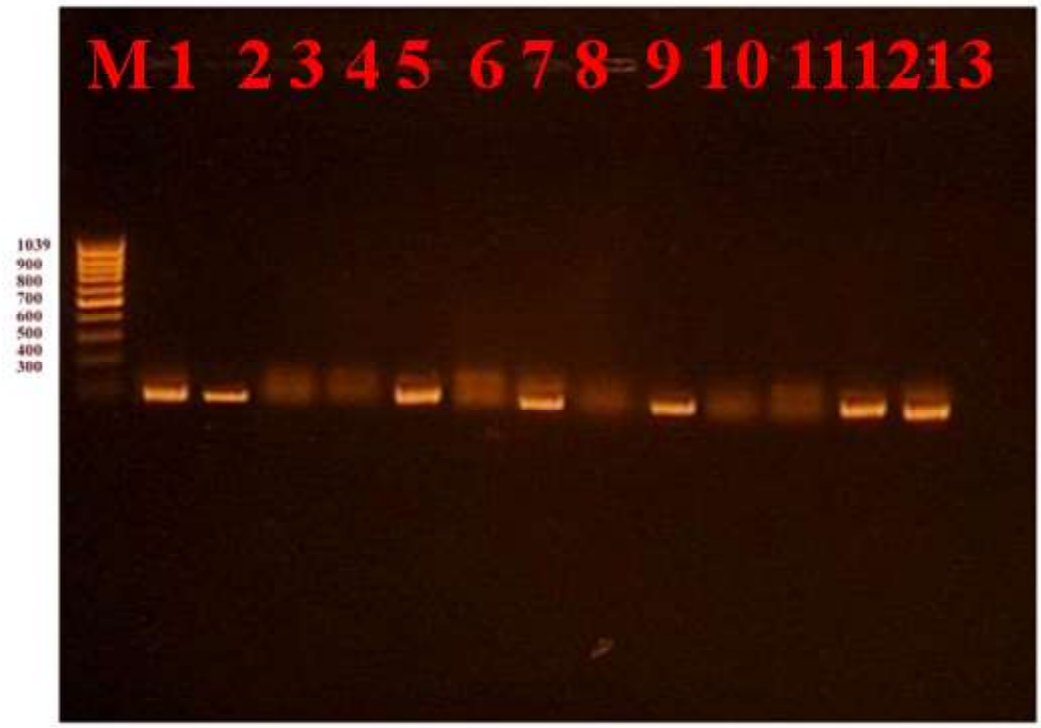

Figure 1. Detection of mecA gene from coagulase positive S. aureus. Lane 1: DNA marker, Lane 2-14: 13 Methicillin susceptible isolates shows mecA

DISCUSSION

Diabetic foot ulcer infections (DFUIs) are serious complications of diabetic mellitus and are caused by a variety of microorganisms particularly *S. aureus* which have strains that are resistant to many of the antibiotics in common use, making the treatment procedures complicated and costly.[30] The findings of the present study also underline the seriousness of the DFU infections as they reveal the nature and characteristics of the bacterial isolates from a total of 300 pus samples collected from DFUIs of type II diabetes patients. Based on detailed analyses, the bacterial isolates from the DFU pus samples were identified and belonged to *S. aureus*, *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Pseudomonas* sp., and *Citrobacter* sp., with 65.3% Gram negative and 34.4% Gram positive bacteria, a clear indication that the DFUIs are polymicrobial. Similarly, Tiwari et al.[31] investigated 62 cases of DFUIs and isolated 82 bacteria wherein they found that the percentages of Gram negative and Gram-positive isolates were 68% and 32%, respectively. The current study was correlated with a previous report of Mutonga et al.[32] in whose study 80 swabs were collected and who found that the percentages of the Gram negative and Gram-positive populations were 65% and 29%, respectively. Among them, 16% were *S. aureus*, 15% *E. coli*, 11% *Proteus mirabilis*, 7% *Klebsiella pneumoniae* and 7% *Pseudomonas aeruginosa,* indicating that *S. aureus* is the most frequently isolated organism in DFUs. Many other studies have also reported that *S. aureus* is an important agent causing DFU, in line with the present study which reports its presence in 34.4% of patients.[33,34]

The antibacterial susceptibility patterns are helpful for recommending suitable antibiotics for the treatment of DFUIs. In this study, all 31 coagulase-positive *S. aureus* were susceptible to novobiocin and all 73 coagulase-negative *S. aureus* were susceptible to methicillin. An investigation by Mergenhagen et al.[35] determined that the prevalence of *Staphylococcus aureus* isolated from patients with DFUIs was 89.2%, with 7.5% of MRSA and 24.8% of methicillin-susceptible *Staphylococcus aureus*. Recently, Anafo et al.[36] investigated the variety of bacteria in 100 patients with DFUIs in Ghana and found that *S. aureus* was the most prevalent bacterium showing resistance to penicillin (100%), tetracycline (47.4%), cotrimoxazole (42.1%) and so on.

Nowadays, the genotypic method such as PCR plays a vital role in the detection of genes involved in the resistance mechanism. In the current study, the *mecA* genes which are responsible for methicillin resistance were detected in *S. aureus* using PCR. Among the 13 isolates which were resistant to methicillin, 7 isolates showed the presence of *mecA* genes after PCR amplification and the remaining MRSA may have other genes such as *mecC*. Our findings were correlated with an earlier report by Anwar et al.[37] wherein they investigated 46 samples for the detection MRSA and predicted that 45.8% were MRSA. PCR showed the presence of *mecA* in 41.6% of MRSA.

There are many limitations for the present study, essentially to be examined at the time of interpreting its findings. Firstly, the specimen collection using swabs has a demerit that it cannot isolate the bacterial pathogens from the inner parts of the ulcers such as the bones. Secondly, the antibiotics that were being received by the patients in the hospital facility were also not considered. Still, the present investigation report affords important basic information for future studies as a thorough knowledge of the microbiology of DFUIs is important in monitoring the treatment and managing the adverse effect of antimicrobial resistance among high risk diabetic patients. Further studies are recommended which should include samples from different levels of skin lesions in a larger number of patients and analyse the virulence factors.

CONCLUSION

This study demonstrated that the 13 methicillin-resistant isolates were analysed for the presence of the *mecA* gene using PCR indicating

that 7 (53.8%) isolates had the *mecA* gene. Overall, the results suggest that DFU infections are polymicrobial in nature and comprise *S. aureus* as the dominant bacterial pathogen.

CONFLICT OF INTEREST

The authors declare that the present study was performed in the absence of any conflict of interest.

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CONTRIBUTORS

PK and MMP: concept development; PK, SSSR and MMP: work design and supervision; PK and MMP: sampling, processing, identification, antibiotic susceptibility tests; PK, SSSR, and MMP: PCR, gel electrophoresis, data analysis and interpretation, literature search, writing and critically reviewing the paper. All the authors have read and approved the final manuscript.

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