The Prevalence of Antibiotic Resistance Genes and Toxin Producing Genes in Methicillin Resistant Staphylococcus aureus Isolates from Diabetic Foot Ulcer

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KEYWORDS

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ABSTRACT

Antibiotic resistance in bacterial pathogens coupled with toxin production can have severe impact on health. Diabetic foot ulcer (DFU) is an extreme pathophysiological condition difficult to treat due to bacterial infections. The present study investigates the antibiotic resistance, prevalence of antibiotic resistance genes and virulence genes in one of the most common Gram-positive pathogen Methicillin resistant *Staphylococcus aureus* from DFUs of Indian patients. The samples were collected form DFU using sterile swab, needle aspiration and curetting. A total of 150 Methicillin resistant *Staphylococcus aureus* isolates were tested for antibiotic resistance using various drugs. The prevalence of the genes involved in antibiotic resistance (Mec*A*) and toxin production (Fem*A*, coa, nuc, aro*A*, alpha-toxin, exf*A*, Fab*A*, Fab*B*, and pvl) was studies using PCR analysis. Among 150 Methicillin resistant *Staphylococcus aureus* isolates 100% isolates were resistant to Benzylpenicillin and Cefoxitin.Most of the isolates were sensitive to Linezolid (4%; 6 isolates), Tigecycline (4%; 6 isolates), Teicoplanin (5.33%; 8 isolates), Nitrofutantion (6%; 9 isolates), and Vancomycin (8%; 12 isolates). Mec 1 gene was present in 84 (54%) isolates. Mec*A* gene was present in81(54%) isolates. Among toxin producing genes Fab-A105(70%) was the most prevalent gene. Mec*A* gene was present in 100% isolates resistant to Benzylpenicillin, Cefoxitin, and Levofloxacin. These results suggest that antibiotic resistance pattern of MRSA isolates vary for different drugs. The possibility of the presence of other antibiotic resistance genes and toxin producing genes in Methicillin resistant *Staphylococcus aureus* isolates could not be denied.

Introduction

Antibiotic resistance in the pathogenic bacteria inhabiting diabetic foot ulcers (DFU) is one of the major obstacles in the treatment [1]. As non-cured DFU cases lead to amputation of the respected body part, the patients suffer significant mental, physical, and economic consequences [2,3]. Since the number of diabetic patents is increasing worldwide at alarming rate, the cases of DFUs are expected to increase by 12-25% in forthcoming years [1,4]. Therefore, careful evaluation of pathogenic bacteria inhabiting DFUs and their antibiotic resistance properties need to be studied. Moreover, pathogenic bacteria co-operate with each other, produce toxins and develop antibiotic resistance [5]. Understanding the genetic basis of

antibiotic resistance and toxin production in bacteria could provide an insight into the mechanism of infection and antibiotic resistance [6,7].

Pathogenic infections in DFUs can be composed of single bacterial species or multi-species [5]. Resent review highlighted the presence of some common pathogenic bacteria including *Staphylococcus aureus*, Escherichia coli, etc. in DFU patients in India [1,7]. Moreover, these common pathogens are being reported to be multi-drug resistant [8-11]. Several reports suggest that these pathogens harbour genes involved in drug resistance and toxin production [8,10,12]. For example, methicillin resistant *Staphylococcus aureus* (MRSA) isolated from DFU carry antibiotic resistant MecA and femA genes [13] and toxin producing genes [12]. Staphylococcus aureus is one of the most common pathogenic bacteria capable of overcoming antibiotic treatment or developing drug resistance [14,15]. The bacteria *Staphylococcus aureus* carries a huge array of antibiotic-resistant genes and toxin producing genes [16]; hence, categorized as "priority pathogens" by world health organization [17]. Importantly, *Staphylococcus aureus* is the most common Gram-positive bacteria found in DFUs in Indian patients [1]. There are some studies reporting the occurrence of antibiotic resistant and toxin producing genes in Staphylococcus aureus from India [14]. Considering the ethnic diversity of India and socioeconomic structure of Indian population, there is an urgent need of the studies exploring genetic mechanism of antibiotic resistance in Staphylococcus aureus bacteria from different parts of the country [1,18].

In the present study, we isolated 150 Methicillin resistant *Staphylococcus aureus* isolates from DFU patients from western Indian state Maharashtra and studied their antibiotic resistance pattern. Also, we studied the prevalence of antibiotic resistance genes and the genes involved in toxin production.

Material and methods

The study was carried out at Department of microbiology, Krishna Institute of Medical Sciences Deemed University, Karad, Maharashtra. The study was carried out during September 2017 to December 2021. A total of 150 patients with DFU harbouring Methicillin resistant *Staphylococcus aureus* admitted at Krishna Hospital Karad were included for the present study. For the present study, the patients with Type I and II diabetes mellitus of all the age groups and both sexes were included. Diabetic patients with foot ulcers of Grade I or more were included in the study. Non-diabetic patients with foot ulcer were excluded from the study.

Demographic information of the patients (including sex, age, duration of Diabetes mellitus, duration of diabetic foot ulcer, and the site of the ulcer) were recorded at the time of admission. DFU associated pathophysiological complications such as hypertension (HPT), cardiovascular disease (CVD), neuropathy (NRP), nephropathy (NEP), and osteomyelitis (OST) were recorded. The ulcers were categorized into different grades following the method described previously [19].

Bacterial samples were isolated using sterile swabs, needle aspiration or as curettage of the base of ulcer. The samples were collected in sterile culture tubes and processed for microbiological assessment in Department of Microbiology and genetic study in molecular Lab K.I.M.S. Karad. For laboratory cultureusing blood agar and MacConkey agar plate. Plates were observed for Gram staining and colony morphology to detect *Staphylococcus aureus* in the specimens. Standard bacteriological techniques were used to identify *Staphylococcus aureus*.

The antimicrobial susceptibility test was carried out by the Kirby-Bauer disc diffusion method on Muller Hinton media as per Clinical and Laboratory Standard Institute. Several single antibiotic discs were used such as Benzylpenicillin, Levofloxacin, Cotrimoxazole, Vancomycin, Gentamicin, ciprofloxacin, Ofloxacin, Cefoxitin, Erythromycin, Clindamycin,

Linezolid, Tigecycline, Netilmicin, and Nitrofurantoin. The ruler was used to measure the inhibition zone in millimeters and compared it with the incorporated chart.

Methicillin resistance in *Staphylococcus aureus* was tested on Mueller Hinton agar supplemented with 4% NaCl using 30 µg Cefoxitin discs by Kirby Bauer disc diffusion method following CLSI guidelines.

Table 1: Details of the primers used for polymerase chain reaction for different genes.

Gene	Sequence (5'→3')	Amplicon size
mecA	FP – GTGAAGATATACCAAGTGATT RP-ATGCGCTATAGATTGAAAGGAT	147 bp
femA	FP –CTTACTTACTGGCTG TAC CTG RP -ATG TCG CTT GTT ATG TGC	686 bp
nucA	FP – 5'GCG ATT GAT GGT GAT ACG GTT3' RP -AGC CAA GCC TTG ACG AAC TAA AGC	270 bp
aroA	FP –AAG GGC GAA ATA GAA GTG CCG GGC RP -CAC AAG CAA CTG CAA GCA T	1153 bp
coa	FP – ATA GAG ATG CTG GTA CAG G RP -GCT TCC GAT TGT TCG ATG C	850 bp

Table 2: Details of the PCR reactions performed for each pair of primers.

Gene	Denaturation	Annealing	Extension	Final extension
mecA	95 °C for 30 s 30 cycles	48 °C for 30 s 30 cycles	72 °C for 30 s 30 cycles	72 °C for 10 min 1 cycles
femA	95 °C for 30 s 35 cycles	54 °C for 1min 35 cycles	72 °C for 1 min 35 cycles	72 °C for 10 min 1 cycle
nucA	95 °C for 30 s 35 cycles	54 °C for 1min 35 cycles	72 °C for 1 min 35 cycles	72 °C for 10 min 1 cycle
aroA	95 °C for 30 s 35 cycles	63 °C for 1min 35 cycles	72 °C for 1 min 35 cycles	72 °C for 10 min 1 cycle
coa	95 °C for 30 s 35 cycles	50 °C for 1min 35 cycles	72 °C for 1 min 35 cycles	72 °C for 10 min 1 cycle
pvl	95 °C for 1min 35 cycles	58 °C for 1min 35 cycles	72 °C for 1 min 35 cycles	72 °C for 10 min 1 cycle
Alpha-toxin	95 °C for 1min 35 cycles	57 °C for 1min 35 cycles	72 °C for 1 min 35 cycles	72 °C for 10 min 1 cycle
exfA	95 °C for 1min 35 cycles	53 °C for 1min 35 cycles	72 °C for 1 min 35 cycles	72 °C for 10 min 1 cycle
fabA	95 °C for 1min 35 cycles	61 °C for 1min 35 cycles	72 °C for 1 min 35 cycles	72 °C for 10 min 1 cycle
fabB	95 °C for 1min 35 cycles	61 °C for 1min 35 cycles	72 °C for 1 min 35 cycles	72 °C for 10 min 1 cycle

DNA extraction of MRSA isolates was done using QIAamp DNA Mini kit (Qiagen, USA) following manufacturer's instructions. PCR amplification of the genes involved in antibiotic resistance (Mec A) and toxin production (Fem, Coa, Nuc, Aro, Alpha T, ExfA Fab-A, Fab-B, and pvl) was performed by using specific primers (Table 1). 10 µl of purified PCR product along with 100 bp mw DNA ladder loaded on horizontal agarose gel for electrophoretic separation and stained using ethidium bromide(Table 2). DNA bands were visualized and photographed under UV trans-illuminator ad Gel documentation system (BioRad, USA).

ATCC *Staphylococcus aureus* 29213 was used as a control strain for genetic analysis. Data were presented in tabular and graphical format using Microsoft Excel.

Table 3: Demographic details of patients with DFU

Demographic parameter	**Value**
Sex	
Male	124 (82.66%)
Female	26 (17.33%)
Age of patients	
21-30	2 (1.33%)
31-40	8 (5.33%)
41-50	15 (10%)
51-60	52 (34.66%)
61-70	47 (31.33%)
71-80	18 (12%)
81-90	5 (3.33%)
91+	3 (2%)
Side	
Left	60 (40%)
Right	86 (57.33%)
Bilateral	4 (2.66%)
Duration of ulcer	
1 month	117 (78%)
3 months	27 (18%)
6 months	5 (3.33%)
1 year	1 (0.66%)
Grade of ulcer	
1	22 (14.66%)
2	49 (32.66%)
3	45 (30%)
4	25 (16.66%)
5	9 (6%)
Associated pathophysiological complications	
Hypertension	23(15.33%)
cardiovascular disease	9 (6.0%)
Neuropathy	39 (26%)
Nephropathy	8 (5.3%)
PVD	8 (5.3%)

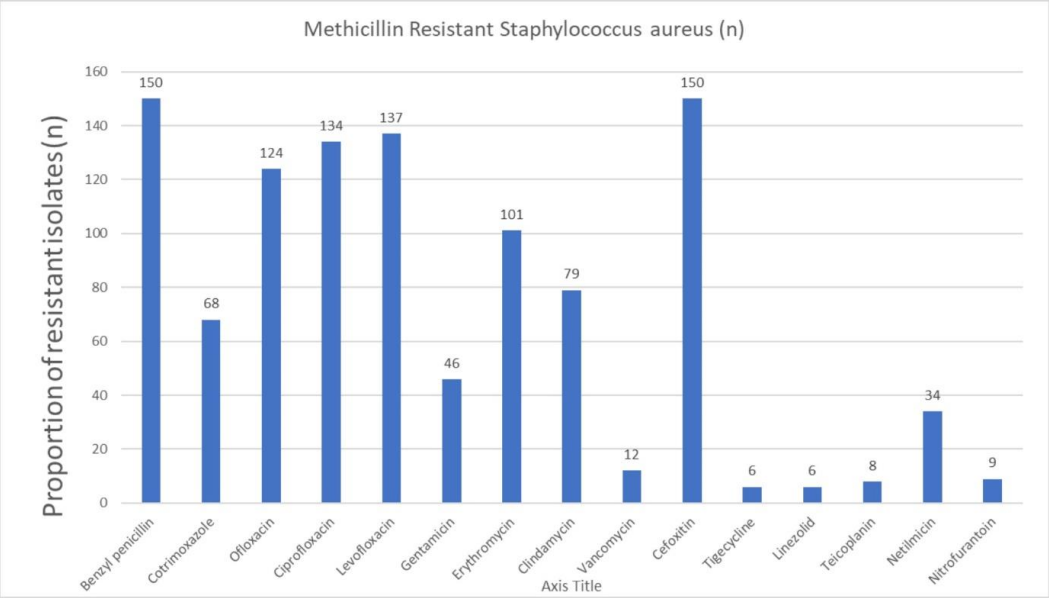
Among 150 patients, 124(82.66%) were males and 26(17.33%) females (Table 3). Most of the patients age ranges between 51-60 (34.66%), 61-70 (31.33%%), and 71-80 (12%). More than 86(57.3%) of the patients had ulcer on right side. Most of the patients had grade 2 ulcer 49(32.66%) and grade 3 ulcer45(30%). Neuropathy 39(26%) was the most common pathophysiological complication associated with ulcer followed by hypertension23(15.33%; Table 3).

In this study, a total of 150 diabetic patients were with MRSA infection. Of the total 150 cases, polymicrobial infections were seen in 79 (52.7%) and monomicrobial infections in 71(47.3%) cases. Among polymicrobial infections, the majority of the cases i.e. 41 (51.9%)

had two isolates and 28(35.4%) had three isolates. Only 10(12.7%) cases had more than three isolates, out of which 9 cases had 4 isolates and one case had 6 isolates.

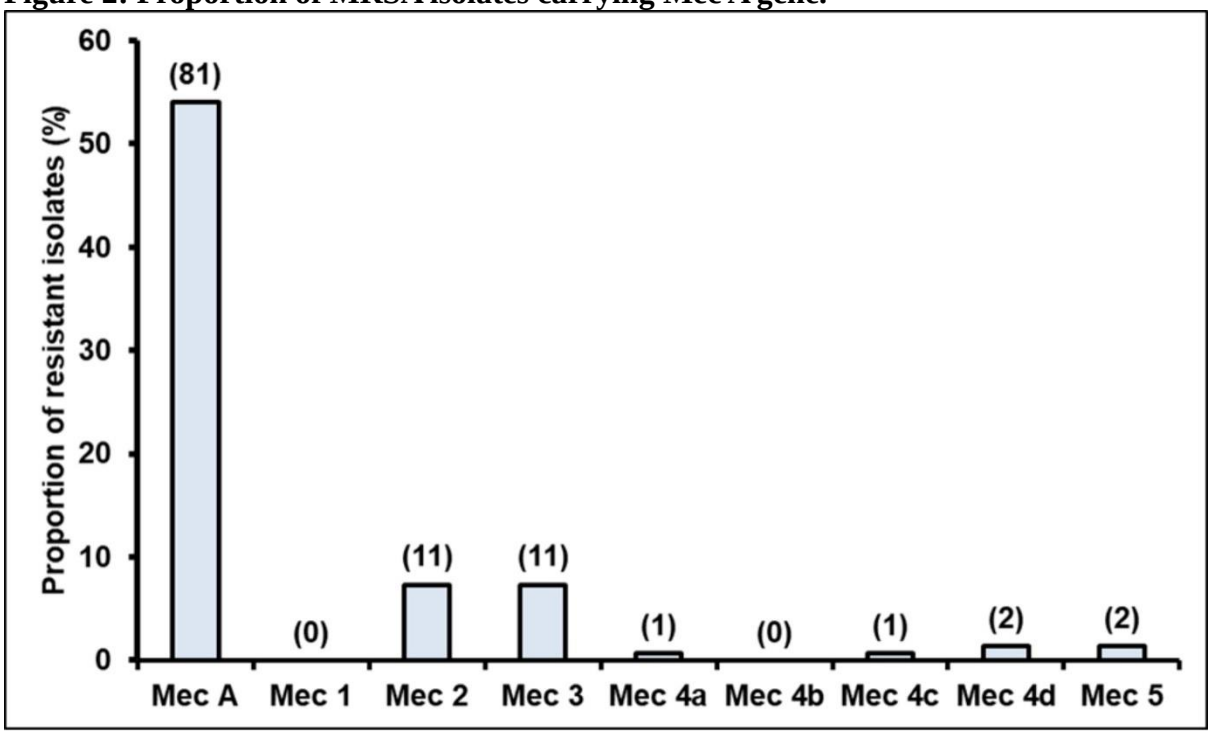
Pseudomonasaeruginosa *32*(11.5%) was the most common bacteria co-habited with *Staphylococcus aureus* followed by *Escherichia coli* *29*(10.4%), *Acenetobacter* 25(9.0%), Klebsiella sp. 26(9.3%), Proteus mirabilis 10 (3.6%), *Streptococcus* 3(1.07%), *Enterococcus 1* (0.35%), *Morgenella* 1(0.35%), *Citrobacter* 1(0.35%), and *Aeromonas* 1(0.35%).

Figure 1: Proportion of antibiotic-resistant MRSA isolates from DFUs.



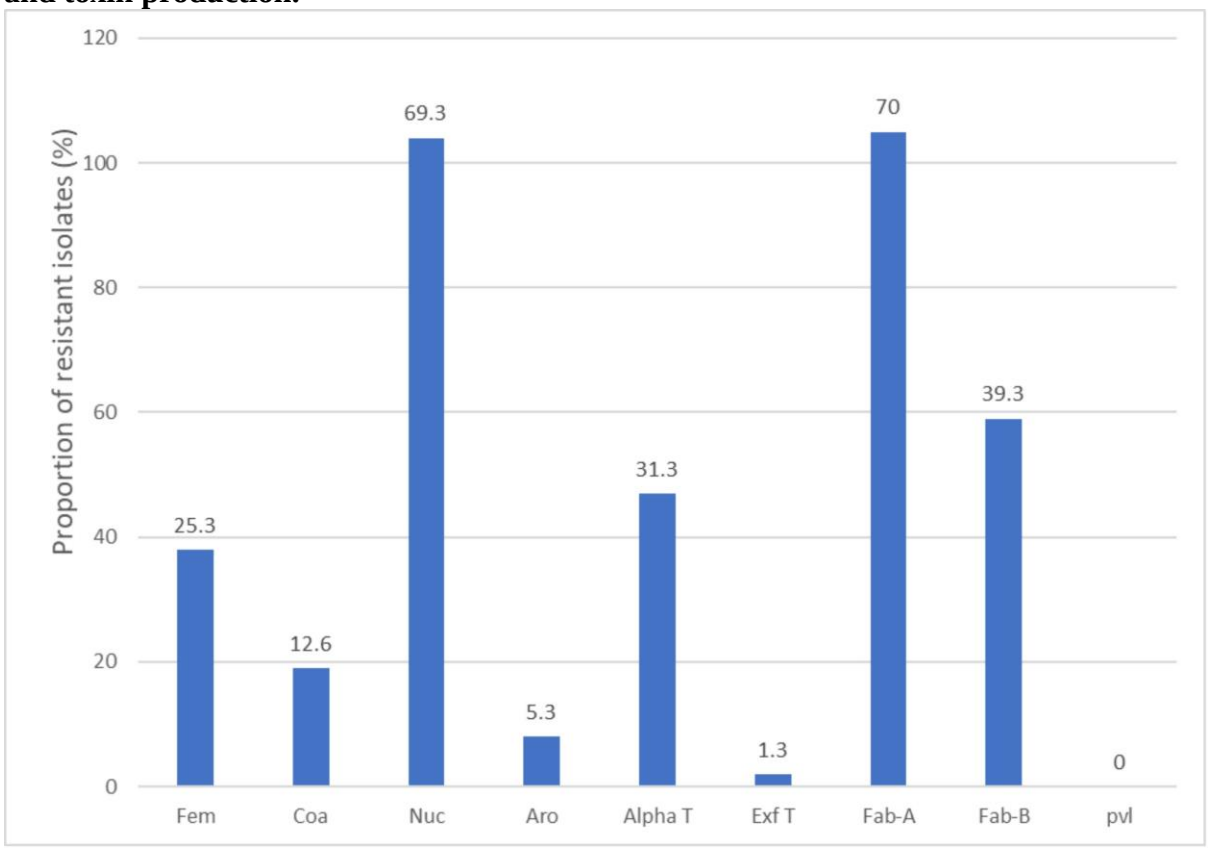
Antibiotic susceptibility testing revealed that all MRSA isolates are resistant to Benzylpenicillin and Cefoxitin followed by Levofloxacin 137(91.33%), Ciprofloxacin 134(89.33%), and Oxacillin124(82.66%; Figure 1). Methicillin resistant *Staphylococcusaureus* isolates were highly sensitive to Linezolid 144(96%) and Tigecycline 144(96%) followed by Nitrofurantoin 141(94%) and Vancomycin 138(92%; Figure 1).

Figure 2: Proportion of MRSA isolates carrying Mec A gene.



Polymerase chain reaction (PCR) analysis of the genes involved in drug resistance revealed that mecA was prevalent in 54% MRSA isolates followed by mec2 (7.33%), and mec3 (7.33%; Figure 2). mecA gene was present in 100% isolates resistant to Benzylpenicillin, Cefoxitin, and Levofloxacin followed by Ciprofloxacin (89.02%), Oxacillin (81.70%), and Erythromycin (63.41%).

Figure 3: Proportion of MRSA isolates carrying different genes involved in virulence and toxin production.



The prevalence of Nuc and Fab-A genes was 69.33% and 70 % in MRSA followed by Fab-B (36.66%), Alpha T (30.66%), and Fem (25.33%; Figure 3). pvl gene was absent in MRSA isolates (Figure 3).

Discussion:

The understanding of genetic basis of antibiotic resistance in pathogenic bacteria is extremely important for designing and developing counter strategies. In the present study, we analyzed the prevalence of the genes involved antibiotic resistance and toxin production in Staphylococcus aureus, one of the common pathogenic bacteria. The results revealed that Methicillin resistant Staphylococcus aureus isolates are drug resistant especially, to Benzylpenicillin and Cefoxitin while they were highly sensitive to Linezolid, Tigecycline, Teicoplanin, and Nitrofurantoin. Similar to the present study, Methicillin resistant *Staphylococcus aureus* isolates were reported to be resistant to Ciprofloxacin, Oxacillin, and Cefotoxitin [20]. Shettigar et al. [12] and Raju et al. [13] reported similar resistance to Ciprofloxacin. Dar et al. [21] reported highest resistance in *Staphylococcus aureus* to Penicillin G. Interestingly, *Staphylococcus aureus* isolates were highly sensitive to Vancomycin in the present study and previously reported studies [13,22]. Contrastingly, Sugandhi & Prakash [20] reported that 98% S. aureus were resistant to Vancomycin. Similar to the present study, Staphylococcus aureus isolates were resistant to Erythromycin (61%), a

macrolide antibiotic. However, Aggarwal et al. [8] Shettigar et al. [12] reported that *Staphylococcus aureus* was highly resistant to Erythromycin. There results suggest that antibiotic resistance pattern in Staphylococcus aureus varies in different studies owing to the differences in the geographical locations. In the present study, we used antibiotics of diverse classes. The results revealed that Methicillin resistant *Staphylococcus aureus* isolates were resistant to β lactum, Penicillin G, Quinolone, and macrolide antibiotics. However, MRSA isolates were highly sensitive to Oxazolidinones, Glycopeptide, Glycycyline, and Nitrofuran antibiotics. These results will be helpful for further investigations on the genetic mechanisms of antibiotic resistance in *Staphylococcusaureus*.

Antibiotic resistance in pathogenic bacteria is determined by the genetic makeup [23]. In *Staphylococcus aureus*, several genes involved in antibiotic resistance have been reported [15,24]. In the present study, we show that mecA is the most prevalent gene in MRSA isolated from DFUs. Previous studies also reported the prevalence of mecA gene in *Staphylococcus aureus* [13,20,22]. Moreover, recent studies from India reported the prevalence of the other antibiotic resistance genes (including Fem A, erm, bla, etc.) from *Staphylococcus aureus* [8,10]. Similar to the present study, antibiotic resistance in Methicillin resistant *Staphylococcus aureus* (isolated from DFUs) due to the presence of mecA gene have been reported [8,12,13,20,22]. Considering a wide range of antibiotic resistance genes found in Staphylococcus aureus, involvement of other genes in different antibiotics needs to be investigated. Further, there are possibilities of the presence other genes governing antibiotic resistance in pathogenic bacteria. For example, Aggarwal et al. [8] reported Erythromycin resistance in *Staphylococcus aureus* which could not be correlated with the presence of erm genes suggesting the presence of unknown genes involved in erythromycin resistance.

The investigation of virulent genes or toxin producing genes is important as these genes help bacteria in infecting healthy cells. The bacterium *Staphylococcus aureus* harbour variety of virulent and toxin producing genes [25,26]. The toxins produced by pathogenic bacteria deteriorate DFU wounds and modulate host immune response [27]. The present study revealed that nuc and Fab-A genes were highly prevalent in Methicillin resistant *Staphylococcus aureus*. Previously, Shettigar et al. [12] reported the presence of virulent and toxin producing genes in *Staphylococcus aureus* from DFUs. The combination of antibiotic resistance genes and toxin producing genes in the bacteria inhabiting DFU wounds help their sustainable colonization [28,29]. Therefore, to understand the infection dynamics and the mechanism of antibiotic resistance in pathogenic bacteria, genetic studies are important.

Considering a targeted bacterial pathogen from a particular pathophysiological condition for genetic analysis can be a good strategy for preliminary understanding of the genetic mechanism of antibiotic resistance [30]. The present study reports the prevalence of some genes involved in antibiotic resistance and toxin production in MRSA from DFU.

One of the major limitations of the study is that the study was conducted at a single locality in Maharashtra state of the western part of the country. Secondly, the study used PCR based technique to study genetic background of the antibiotic resistance considering a few genes. Further studies focusing on the genome level analysis of antibiotic-resistant bacteria are necessary for developing strategies for antibiotic treatments for DFUs. In addition, representation of the population of the Maharashtra state need to considered in the future studies to eliminate the influence of the ethnicity.

Conclusion:

The present study reports the antibiotic resistance in *Staphylococcus aureus* along with the prevalence of some antibiotic resistance genes and virulence genes. The results revealed that mecA is the most prevalent antibiotic resistance gene in these isolates. Whereas Nuc and Fab A were the most prevalent virulent genes in aureus isolates. Resistance in Staphylococcus aureus to some antibiotics can be partially correlated with the presence of antibiotic

resistance genes. It seems that there are other unknown genes are involved in antibiotic resistance of *Staphylococcus aureus*. Further genomic studies on the antibiotic resistance in *Staphylococcus aureus* focusing on the mechanism need to be undertaken.

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