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Detection of Methicillin-Resistant *Staphylococcus aureus* in Diabetic Foot Infections

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A B S T R A C T

Diabetes mellitus is a serious public health problem and remains an important cause of morbidity and mortality. The Indian diabetic population is expected to increase to 57 million by the year 2025 and 87 million by 2030. Methicillin-resistant Staphylococcus aureus (MRSA) has emerged as a serious and common problem in patients with diabetic foot Infection. Colonization with MRSA may result in prolonged hospital stay and excessive direct economic cost. Until now few studies are available about methicillin-resistant S. aureus (MRSA) strains isolated in diabetic foot infections in this part of India. Therefore the study was conducted to know the prevalence and antibiotic resistance pattern of MRSA isolated among the diabetic foot Infection. Materials and methods: The study was conducted in the Department of Microbiology, Shri B.M Patil Medical College Hospital and Research center, Vijayapur. A total of 96 patients with history of diabetes which yielded S. aurius were included in the study. Antimicrobial susceptibility testing of the isolates was performed by Kirby Bauer disc diffusion method. MRSA were detected by Cefoxitin Disc Diffusion Test, Oxacillin Disk Diffusion Method and by mecA gene PCR The male DFI patients formed the sources for majority of the isolates with percentage of 55.2%. patients with DFI were more among the elderly people age group of 41-60, followed by 61-8- age group. In the present study, isolation rate of MRSA was 44% in our study. MRSA isolates were more resistant then MSSA isolates. Detection of mecA gene is considered the gold standard for MRSA confirmation. In our study, the PCR detected 44 isolates as MRSA and the 52 isolates as MSSA. Cefoxitin and Oxacillin detected 42 and 35 isolates as MRSA respectively. Diabetic foot infections (DFI) are very serious and life threatening if not treated in time and with proper antibiotics. MRSA are one of the important causes of diabetic foot Infection and hence should be identified early for better outcome. cefoxitin disc is better than oxacillin disc for the detection of methicillin resistance. Results of cefoxitin disc diffusion test are as good as PCR used for mecA gene, and thus the cefoxitin can be used for identification of MRSA in setting where PCR is not available.

Introduction

Diabetes mellitus is a serious public health problem and remains an important cause of morbidity and mortality. (Zaini *et al.,* 2000)The Indian diabetic population is expected to increase to 57 million by the year 2025(Shakil *et al.,* 2010).and 87 million by 2030.(Tiwari *et al.,* 2012)It is well known that patients with poorly controlled DM are at risk of developing diabetic complications such as pedal ulcers with or without gangrene, retinopathy, neuropathy and macro vascular complications.

(Benwan *et al.,* 2012) Diabetic foot infections (DFIs) are common, complex and costly complications of DM. In addition to causing severe morbidities, they account for the largest number of diabetes related hospital inpatient days and are the most common proximate, non-traumatic cause of amputations. (Lipsky *et al.,* 2004)

Foot complications such as foot ulcer constitute a major public health problem and impose a heavy burden on health services. Foot infections are responsible for the majority of diabetes - associated hospital admissions. It was estimated that approximately15% of all diabetics develop foot ulcers and eventually progress to osteomyelitis. (Ramsey *et al.,* 2004)

The impaired micro-vascular circulation in patients with a diabetic foot limits the access of phagocytes, thus favoring the development of an infection. The local injuries and the improper foot wear further compromise the blood supply in the lower extremities.

While the foot infections in persons with diabetes are initially treated empirically, a therapy which is directed at the known causative organisms may improve the outcome. (Citron *et al.,* 2007).

Optimal management of DFIs can reduce the incidence of infection-related morbidities, the need for and duration of hospitalization, and the incidence of major limb amputation. Early identification of lesions, prompt initiation of appropriate antibiotic therapy, aggressive surgical debridement of necrotic soft tissue and bone, and modification of host factors are all equally important for a successful clinical outcome. (Lipsky *et al.,* 2004)

Reports from western countries have found that Staphylococcus aureus and β-haemolytic streptococci are the main causative pathogens (Dang *et al.,* 2003). In appropriate antibiotic usage contributes to the increasing prevalence of multidrug-resistant organisms, notably methicillin-resistant S. aureus (MRSA).

Methicillin-resistant Staphylococcus aureus (MRSA) has emerged as a serious and common problem in patients with diabetic foot ulcers. Infection⁄ colonisation with MRSA may result in prolonged hospital stay and excessive direct economic cost. (Tentolouris *et al.,* 2006)

Until now few studies are available about methicillin-resistant S. aureus (MRSA)strains isolated in diabetic foot infections in this part of India. Therefore the study was conducted to know the prevalence and antibiotic resistance pattern of MRSA isolated among the diabetic foot Infection.

Materials and Methods

The study was conducted in the Department of Microbiology, Shri B.M. Patil Medical College Hospital and Research center, Vijayapur. A total of 96 patients with history of diabetes which yielded S. aurius were included in the study after approval of institutional Ethics Committee. Samples were collected from the deeper portion of the ulcers by using 2 sterile swabs which were dipped in sterile glucose broth. The samples were

collected by making a firm, rotatory movement with the swabs (Shanmugan *et al.*, 2013).

Statistical analysis

Values were expressed in terms of Mean ± SD. Analysis was done by using SPSS software version 16. P≤0.05 was considered statistically significant.

Inclusion criterion

Patients with history of diabetes which yielded *S. aurius* were included in the study

Exclusion criterion

Patients without history of diabetes were excluded from the study Specimens were screened by preliminary Gram's stain andthen inoculated on 10% sheep blood agar and Mac Conkey's agar. *S. aureus* were identified by conventional techniques. Antimicrobial susceptibility testing of the isolates was performed by Kirby Bauer disc diffusion method using following discs. penicillin-G(10unit); cloxacillin (30µg); cephalexin (30µg); cefuroxime(30 µg); tetracycline (30µg) ; erythromycin (15µg); gentamicin (10µg); ciprofloxacin (5µg); pefloxacin (5µg); Cefoperazone / salbactam (75µg/30µg); azithromycin(15µg); linezolid (15µg). Vancomycin(30µg); piperacillin / tazobactam (100µg/10 µg); amoxicillin /clavulanic acid (20 µg /10 µg).

The data were recorded and analyzed at the completion of the study as per recommendations of the CLSI. (M100-S21, *Clinical and Laboratory Standards Institute*, 2011)

Detection of MRSA

The cefoxitin disc diffusion test

The Cefoxitin disc diffusion method was carried out on Mueller-Hintonagar by using a 30 µg cefoxitin disc. Inoculum was prepared and compared with 0.5McFarland turbidity constant. Mueller-Hintonagar was inoculated and excess was removed. Cefoxitin 30mcg discs were applied with force ps and pressed gently to ensure even contact with the medium. The plates were incubated for 18–24hours at37°c. Interpretation was done using the Kirby-Bauer charts. An inhibition zone diameter of ≤ 21 mm was reported as methicillin resistant.(Isenberg *et al.*, 2004)

The Oxacillin Disk Diffusion Method

The Oxacillin disk (1µg) diffusion method was carried out on Mueller-Hintonagar which was supplemented with 4% NaCl to detect MRSA according to the CLSI guide lines. The isolates were considered as resistant when the diameter of inhibition was ≤10 mm. (Brown *et al.*, 2005)

Genotypic detection of MRSA by PCR (mecAgene) (Vanpelt *et al.*, 2008)

DNA Extraction Procedure was done by Modified Proteinase-K method. MRSA strains were amplified by conventional PCR. Following set of PCR primers were used which were specific to Methicillin resistant *S. aureus* (Boucher *et al.*, 2008).

Forward Primer

5'-TGC TATCCA CCC TCAAAC AGG-3' Reverse Primer: 3'-AAC GTTGTAACCA CCCCA AGA-5' AMPLIQON RED 2X Master mix was used which contain following reagents Tris-HCL pH 8.5, (NH4)2SO4, 3mM MgCl2, 0.2% Tween 20, 0.4mM of each dNTP, 0.2 units/µl Ampliqon Taq DNA Polymerase. The PCR conditions were as follows:

Initial denaturation (940C, 5min), Denaturation (940C, 1min),

Annealing (500C,1 min),
Extension (720 C, 2 min),
Final extension (72°C for 5 min).

Reagents with their company names: PCR.
Master mix: Ampliqon Oligonucleotide.

Primers

Bio serve India pvt. Ltd.

Instruments

Thermal cycler: Applied Biosystems, USA Electrophoresis apparatus: Bio bee Tech, Bangalore. Gel Documentation system: Major Science, USA.

The PCR was carried out for MRSA strains with MRSA specific primer set. After PCR, the agarose gel electrophoresis was done where PCR amplified products were run on a 2% agarose gel.

After running the electrophoresis, the amplified products will get separated on the gel according to the product size which was determined while choosing a primer.

We had chosen a primer set which gives amplified product of size 280 base pair.

So the well which gives DNA band of 280 base pair is considered positive, whereas the well which does not have any DNA band is indicated as negative.

The size or the position of the DNA band can be known by running the DNA ladder simultaneously with each gel

Results and Discussion

As shown in table 1 the male DFI patients formed the sources for majority of the isolates with percentage of 55.2. As shown in table 2,patients with DFI were more among the elderly people age group of 41-60, followed by 61-8- age group. As shown in table3, in the present study, isolation rate of MRSA was 44% in our study.

Table.4 shows that Detection of mecA gene is considered the gold standard for MRSA confirmation. In our study, the mecA gene PCR detected 44 isolates as MRSA and the 52 isolates as MSSA. Cefoxitin and Oxacillin detected 42 and 35 isolates as MRSA respectively

Table.1 Distribution of patients according to sex

SEX	N	%
Female	43	44.8
Male	53	55.2
Total	96	100

Table.2 Association of age with sex among patients with DFI

AGE (YRS)	Male		Female	
	N	%	N	%
1-40	6	11.3	5	11.6
41-60	33	62.6	25	58.2
61-80	12	22.6	8	18.6
Above 80	2	3.7	5	11.6
	53	100	43	100

Table.3 Distribution of MRSA and MSSA among patients with DFI

S. aureus	**N**	**%**
MSSA	5	54
MRSA	4	46
Total	9	100

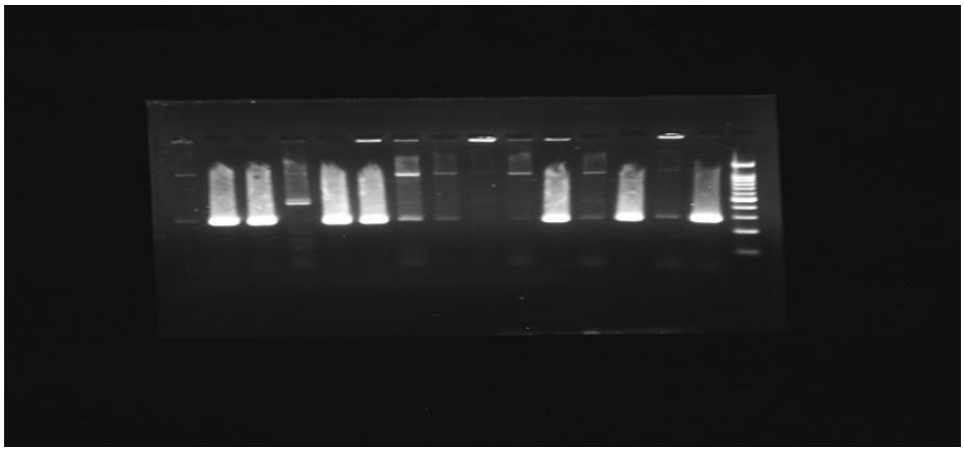
Table.4 Comparative results of phenol typic methods with PCR

TEST METHODS	MRSA	Sensitivity	Specificity	PPV	NPV	Accuracy
Oxacillin	35	79.5%	94.9%	93.7	83.1	87.5%
Cefoxitin	42	95.4%	100.0%	100.0	96.1	97.9%
PCR	44	100.0%	100.0%	100.0	100.0	100.0%

Table.5 Comparison of resistance pattern of MRSA and MSSA among patients of DFI

Antibiotic susceptibility pattern	**MRSA (N=44)**		**MSSA (N=52)**		**pvalue**
	R	**%**	**R**	**%**	
PENICILLIN-G	43	97.7	45	83.3	0.048*
EYTHROMYCIN	26	59	33	63.5	0.661
TETRACYCLINE	12	27.3	8	15.4	0.153
CEPHALEXIN	31	70.5	24	46.2	0.016*
CLOXACILLIN	20	45.5	17	32.7	0.200
PEFLOXACIN	35	79.5	31	59.6	0.036*
PIPERACILLIN/TAZOBACTAM	17	38.6	8	15.4	0.010*
CEFOPERAZONE /SULBACTAM	17	38.6	15	28.8	0.311
GENTAMICIN	13	29.5	11	21.2	0.344
CIPROFLOXACIN	35	79.5	31	59.6	0.036*
AMOXICILLIN/CLAVULANATE	32	72.7	29	55.8	0.085
CEFUROXIME	21	47.7	14	26.9	0.035
AZITHROMYCIN	21	47.7	18	34.6	0.192
VANCOMYCIN	9	20.5	6	11.5	0.231
LINEZOLID	8	18.2	3	5.7	0.057

Note:* significant at 5% level of significance ($p<0.05$)



Results of *mecA*gene(left to right)
Lane1: Molecular weight markerLane2: MRSA ATCC43300
Lane3: MSSA ATCC25923
Lane4, 6, 11,12,14 and 15: MRSA isolates from clinical samples (280 BP)
Lane5, 7-10, 13, and 16: MSSA isolates from clinical sample

Diabetic foot ulcers are more prone to bacterial infections that spread rapidly, leading to irreversible tissue damage. Complications usually begin with an unrecognized foot ulcer in a patient with an insensate foot which gets infected, leading to significant morbidity and lower extremity amputations. Patterns of microbial infection are not consistent in patients with diabetic foot infections and therefore repeated evaluation of microbial characteristics and their antibiotic sensitivity is necessary for selection of appropriate antibiotics. Progression of infection in diabetic foot is a result of suppressed immune status, delayed diagnosis, underestimation of extent of infection, or suboptimal (if not inappropriate) antimicrobial therapy. (Tiwari *et al.,* 2012)

Diabetic patients often have chronic non-healing foot ulcers due to several underlying factors such as neuropathy, high plantar pressures and peripheral arterial disease. Such chronic long-standing ulcers are more prone for infection which further delays the wound healing process.(Sivaraman *et al., 2011*) as shown in table1, in the present study, MRSA was pre dominantly isolated from males (55%) which correlated with study of (Raja *et al.,* 2007).

As shown in table 2, patients with DFI were more among the elderly people age group of 41-60, followed by 61-80 age group this finding correlates well with (Shanmugam *et al.,* 2013).who reported a similar findings.

Management of diabetic foot infections usually requires combination therapy with surgical drainage and debridement or osseous resection. The choice of antibiotic therapy is influenced by the sensitivity of the encountered bacterial pathogens.

Accurate microbiological working is imperative to the choice of appropriate antibiotic therapy for diabetic foot infections. Several drugs have been used to treat non-limb-threatening infections including beta-lactamase inhibitors, third generation cephalosporins, aminoglycosides, ampicillin, penicillin, quinolones, piperacillin - tazobactam and linezolids. Third-generation cephalosporins are not active against enter ococci and an aerobes, while fluoroquionol one shave low activity against streptococci

and anaerobes (Raja *et al.,* 2007).

The antibiogram results in this study suggest that pathogens remain sensitive to a number of agents. Imipenem was equally effective against Gram-negative bacilli and Gram-positive cocci. Vancomycin was found to be the most effective drug overall against Gram-positive organisms. These findings are consistent with a previous study. Our findings illustrate that antimicrobial therapy needs to be selected based on actual culture findings and antimicrobial sensitivity patterns of isolates. (Raja *et al.,* 2007)

Antibiotic susceptibility pattern revealed a high resistance to routinely used antibiotics. Resistance to quinolones i,e. ciprofloxacin and pefloxacin were high in this study. This is comparable to the study done by (Sanjana *et al.,* 2010) in Nepal. Resistance to cephalexin was also much higher in this study. This is consistent with to the study carried out by (Sanjana *et al.,* 2010) who reported the similar resistant rate to cephalexin. (Majumder *et al.,* 2001).also revealed that resistance to various antibiotics with methicillin resistant strains was s higher in comparison to methicillin-sensitive isolates.

Factors responsible for to drug resistance in MRSA are as follows. Antibiotics are available without prescription at drug stores or even at general stores and injudiciously used in communities, animal husbandries, and fisheries and use of allopathic drugs by traditional practitioners. (Metri *et al.,* 2014)

Detection of mecA gene is considered the gold standard for MRSA confirmation. In our study, the mecA gene PCR detected 44 isolates as MRSA and the 52 isolates as MSSA. Recent studies including our s indicate that cefoxitin disc diffusion test is better than most of the phenotypic methods like oxacillin disc diffusion and oxacillin screen agar testing and is now an accepted method for the detection of MRSA by many reference groups including CLSI. The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by S. aureus. (Anand *et al.,* 2003) T

his higher sensitivity to cefoxitin can be explained by the increased expression of the mecA-encoded protein PBP2a, cefoxitin being an inducer of the mecA gene. (Anand *et al.,* 2003) Our study reveals that cefoxitindisc is better than oxacillin disc for the detection of methicillin resistance.

Results of cefoxitin disc diffusion test is as good as PCR used for mecA gene, and thus the cefoxitin can be used for identification of MRSA and the test can be used as cost effective method when compared PCR for detection of MRSA .

Diabetic foot infections (DFI) are very serious and life threatening if not treated in time and with proper antibiotics. MRSA are one of the important causes of DFI, and hence should be identified early for better outcome. Cefoxitin disc is better than oxacillin disc for the detection of methicillin resistance. Results of cefoxitin disc diffusion test are as good as PCR used for mecA gene, and thus the cefoxitin can be used for identification of MRSA in setting where PCR is not available.

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