Available online at **www.ijmrhs.com**

ISSN No: 2319-5886

International Journal of Medical Research & Health Sciences, 2016, 5, 4:16-21

*RPO*B gene mutation in rifampicin resistant MRSA from foot infections of patients having diabetes mellitus

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ABSTRACT

Staphylococcus aureus is an opportunistic bacterial pathogen associated with asymptomatic colonization of the skin and mucosal surfaces of normal humans. Staphylococcus aureus isolates are often multidrug resistant. Rifampicin is a valuable antibiotic for Staphylococcal infection and it is effective in combination therapy, especially for deep-seated infections, owing to its excellent pharmacokinetic properties and bactericidal activity. Rifampicin resistance, however, is frequent among methicillin-resistant Staphylococcus aureus (MRSA) in several countries. Prevalence of this bacterium was about 52% in patients with diabetic mellitus. Mutations in the rifampin resistance-determining (Rif) regions of Staphylococcus aureus were studied by gene amplification and sequencing methods. Five mutational changes were recorded in cluster I, II and III in rpoB gene of rifampicin resistant Staphylococcus aureus.

Keywords: *Staphylococcus aureus*; RIF cluster; *rpo*B *gene*; rifampicin.

INTRODUCTION

Staphylococcus aureus is a common nosocomial pathogen. *Staphylococcal* infection markedly increases the morbidity and mortality in hospitalized patients [17]. The organism has emerged as one of the important and major threat to human community due to rise in antibiotic resistance [26]. Staphylococcus causes frequent infections on prosthetic devices, osteomyelitis, and endocarditis [10]. Multi resistance has become a common feature in *Staphylococcus aureus* [3]. Methicillin-resistant *Staphylococcus aureus* (MRSA) has now emerged as a widespread cause of community infections. The growing problem in the Indian scenario is that MRSA prevalence has increased from 12% in 1992 to 80.83% in 1999 [18]. It is responsible for a wide spectrum of infections and has a remarkable genetic versatility which allows them for the adaptation to multiple antibiotics. *Staphylococcus aureus* infections may occur with greater frequency among patients with diabetes mellitus [8]. Isolation of *Staphylococcus aureus* from diabetic lesions has been studied extensively [24]. Most of the isolates from diabetic foot lesions are found to be resistant to antibiotics [22] and it has been classified as one of the three major public health threats of the 21st century [23]. Therefore, antibiotic options in the treatment of diabetic foot ulcers against these organisms are extremely limited.

Vancomycin is used against *Staphylococcus aureus* when β-lactams antibiotics are inappropriate and also due to poor tissue diffusion and moderate bactericidal activity [5]. Vancomycin is often used in combination with rifampicin for deep-seated infections [25]. Rifampicin is a broad-spectrum antibiotic which is used in most of the cases as a combination therapy with other therapeutic agents as rifampicin reveals strong activity, good tissue penetration and strong activity against dormant or slow-growing bacteria [9,21]. Rifampin resistance in *Staphylococcus aureus* is due to the amino acid alterations in the domain region of RNA polymerase leading to a reduced affinity of the enzyme for the antibiotic [20]. It interacts with bacterial DNA depended RNA polymerase

beta subunit coded by *rpo*B gene [4]. Previous studies on other bacteria indicate that point mutations in RNA polymerase beta subunit are responsible for rifampin resistance in bacteria [6].

The study aims at determining the level of rifampicin resistance in *Staphylococcus aureus* and to investigate the relationship between genetic alterations in the RNA polymerase beta subunit gene and the level of rifampicin resistance in *Staphylococcus aureus*.

MATERIALS AND METHODS

Sample collection and isolation

Swab samples of diabetic patients with foot ulcers were collected from hospitals in and around Chennai. The collected samples were stored in sterile container and transported to the laboratory within two hours of collection and stored in 4 °C, till the processing. The collected swabs were directly inoculated into nutrient broth and incubated overnight at 37 °C. The isolates present in the swab samples were allowed to grow, from which drug resistant *Staphylococcus aureus* were screened and analyzed.

Isolation of *Staphylococcus aureus*

The isolation of *Staphylococcus aureus* was performed using Mannitol salt agar (Pancreatic digest of casein – 5 g, pancreatic digest of animal tissue – 5 g, Beef extract – 1 g, D Mannitol – 10 g, sodium chloride – 75 g , phenol red – 25 mg, agar – 15 g and distilled water – 1000 ml. pH of the media was adjusted to 7.4). To this sterile media a loop of overnight incubated culture from nutrient broth was streaked on to mannitol salt agar plates and incubated overnight for the enumeration of *Staphylococcus aureus*.

Biochemical characterization

The isolates from the mannitol plate were subjected for biochemical characterization. Microbial and biochemical parameters such as Gram staining, catalase, oxidase, indole, coagulase, nitrate reduction, methyl red, Voges-proskaur, glucose, mannitol and sucrose utilization were performed to confirm the identity of the organism.

Determination of multiple antibiotic resistances

Antibiograms of all coliforms were determined on Muller Hinton agar (Hi-Media Pvt. Ltd. Mumbai) using Kirby-Bauer disc diffusion method [7] to assess the resistance of *Staphylococcus aureus* to various antibiotics. The antimicrobial agents were chosen on the basis of their importance in treating human infections caused by gram positive bacteria of the family Staphylococcaceae. The antibiotics included for the study are ampicillin, penicillin, cephalexin, gentamicin, streptomycin, tetracycline, methicillin and rifampicin. Multiple antibiotic resistances (MAR) index [12] was calculated by the formula [11].

$$\text{MAR Index} = y/nx$$

where y = total number of resistance scored; n = number of isolates; x = total number of antibiotics tested.

Detection of rifampicin resistance-associated mutations

The chromosomal DNA of the rifampicin resistant *Staphylococcus aureus* was extracted using standard phenol:chloroform method [27]. An internal sequence of gene *rpo*B of 460bp was amplified using polymerase chain reaction (PCR). The amplification was carried out in rifampicin resistant *Staphylococcus aureus* (rifampicin and methicillin resistant), rifampicin resistance-associated mutations were studied using primers *rpo*B1 (5′-ACCGTCGTTTACGTTCTGTA-3’) and *rpo*B2 (5′-TCAGTGATAGCATGTGTATC-3’), [14] which amplified a 460 bp sequence of *rpo*B gene, encompassing majorly clusters I and II. Amplification was carried out in a 20 µl volume containing 0.3 µM of each primer, 0.2 mM deoxy nucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 1 µl of template DNA sample and 1 U of Prime TaqDNA polymerase (Genetbio, Korea). The reaction tubes were subjected for thermal cycling reactions consisted of an initial denaturation (5 min at 94 °C) followed by 32 cycles of denaturation (1 min at 94 °C), annealing (45 s at 56 °C) and extension (1 min at 72 °C), with a final extension (10 min at 72 °C). The PCR product was purified (QIAquickPCR purification kit, Qiagen, Madrid, Spain) and analyzed by DNA sequencing. The nucleotide sequences obtained were compared to the *rpo*B wild type sequence from *S. aureus* subsp. (GenBank accession number: X64172.1) using the EMBOSS.gui and BLAST tool.

Protein modeling building of *rpo*B gene

The protein models of wild-type and mutated *rpo*B were predicted by comparing the *Staphylococcus aureus rpo*B sequences with the structure of *Escherichia coli* K-12 RNA polymerase (3IYD) using Modeller 9.10 [1,2,13,15] . The models were further refined using Ramachandran plots. The mutated and non-mutated *rpo*B protein structures were superimposed using Accelrys Discover Studio 2.5 to determine the mutations in rifampicin resistance-determining region (RRDR).

RESULTS

Swab samples of diabetic patients were collected from hospitals in and around Chennai. The isolation and biochemical studies were performed and resulted in the characterization of *Staphylococcus aureus*. Table 1 indicates the biochemical and microbial results for the isolated strain. Total of 13 *Staphylococcus aureus* isolates were isolated from 25 patients and prevalence of *Staphylococcus aureus* was about 52%.

Table 1: Microbial and biochemical features

Test	Strain 1 to 13
Gram staining	+ve
Catalase	+ve
Oxidase	-ve
Coagulase	+ve
Indole	-ve
MR	+ve
VP	+ve
Citrate	+ve
Nitrate reduction	+ve
glucose	+ve
Mannitol	+ve
Sucrose	+ve

Table 2: Mutations found in the *rpo*B gene of MRSA isolate

S. No.	Nucleotide position	Nucleotide mutation	Amino acid position	Amino acid substitution	Secondary structure		
		Normal	Rif resistant		Normal	Rif resistant	
1	1366	TCT	ACT	456	S	T	Helix
2	1441	CAT	AAT	481	H	N	Turn
3	1586	TCA	TTA	529	S	L	Sheet
4	1674	GAT	GAA	558	D	E	Sheet
5	1675	CAA	AAA	559	Q	K	Sheet

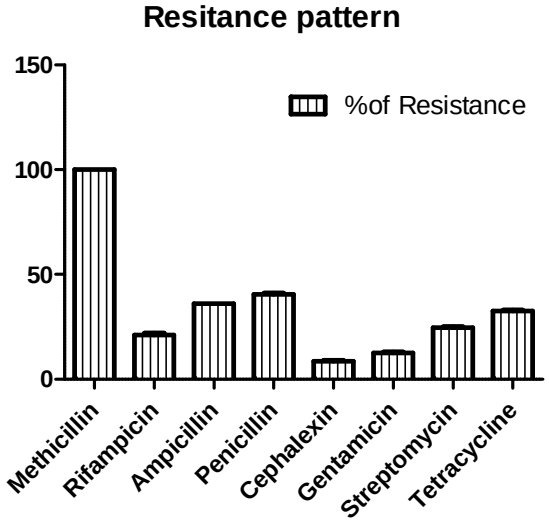

Figure 1: Percentage of antibiotic resistance in *Staphylococcus aureus*

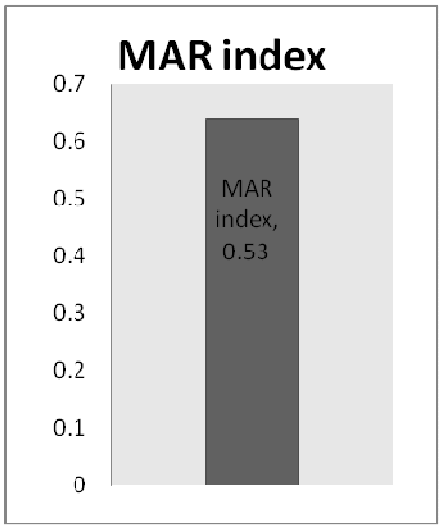

Figure 2: Multiple antibiotic resistance (MAR) index of *Staphylococcus aureus*

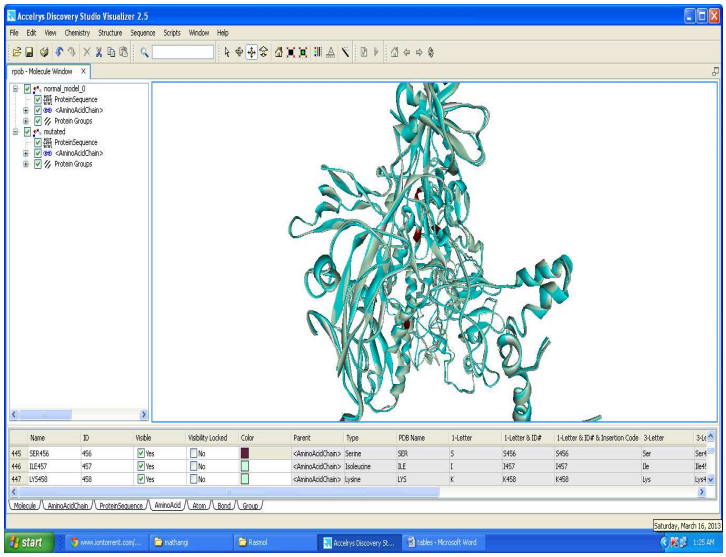

Fig 3: Structural implication of the mutations in *rpo*B gene of *Staphylococcus aureus*

Figure 1 indicates the percentage of antibiotic resistance of isolated *Staphylococcus aureus*. The results indicate that all isolates were methicillin resistant; out of these MRSA strains 20%, 36%, 40%, 8%, 12%, 24% and 32% were resistant for rifampicin, ampicillin, penicillin, cephalexin, gentamicin, streptomycin and tetracycline respectively. Figure 2 indicates the multiple antibiotic resistance (MAR) index value at 0.53 for the isolated *Staphylococcus aureus*, which indicates the MAR index for the isolates strains at >0.2. Figure 3 indicates the superimposed protein structure of mutated and non-mutated *rpo*B gene and the structural mutations in rifampicin resistance-determining region (RRDR) was analyzed.

The mutations in the rifampicin resistance-determining region of *rpo*B gene were studied in MRSA strain and mutational changes in the *rpo*B region are detailed in Table 2. This indicates 5 mutational changes at amino acid position 456, 481, 529, 558 and 559. Structural analysis of *rpo*B revealed 2 Mutations in cluster I (amino acid positions 450–488), 1 mutation in cluster II (amino acid positions 515–530) and 2 mutations in external domain region (546-613). The amino acid substitutions in domain regions confirmed rifampicin resistance in *Staphylococcus aureus*.

DISCUSSION

The multi-resistant natures of most MRSA strains found in hospitals symbolize a therapeutical challenge for treating serious MRSA infections. Rifampicin is an antibiotic of substantial interest in the rise of MRSA infection. Due to the rapid development of resistance, rifampicin is not used as monotherapy, but it has been used in combined therapy for a wide range of staphylococcal infections [19]. All rifampicin-resistant *Staphylococcus aureus* in our study had a high level of Methicillin, penicillin and ampicillin resistance. MAR index analyses of the *Staphylococcus aureus* illustrate value of 0.53. MAR index higher than 0.2 has been said to be an indication of isolates originating from an environment where antibiotics were often used [16]. The MAR values can however be viewed as an indication of the extent of microbial exposure to antibiotics used within the community. Rifampicin resistance is due to the amino acid substitutions in the three clusters (I, II and III) of the rifampicin-binding site of RNA polymerase beta subunit, and the genetic determinants for most rifampicin-resistant *Staphylococcus aureus* isolates identified are point mutations that have been mapped in clusters I and II [6,20] and. Isolate belonging to multi-resistant *Staphylococcus aureus* showed rifampicin resistance and the amino acid substitutions at 481 H/N, 456 S/T, 529 S/L, 558 D/E and 559 Q/K were noted in the *rpo*B gene. The resistance levels are dependent on both the location and the nature of the amino acid substitution. Rifampicin resistance determining region of *Staphylococcus aureus* spans round 463–550 amino acids [28]. All the mutations except 456 S/N were located with the RRDR region of *Staphylococcus aureus* (Table 2). Mutation at the regions 481 H/N and 529 S/L were reported previously in rifampicin resistant *Staphylococcus aureus* [28].

CONCLUSION

From the analysis we have established that rifampin resistance in *Staphylococcus aureus* is probably due to mutations in the Rif region of the *rpo*B gene and the resistance levels are dependent on both the location and the nature of the amino acid substitution.

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