Impact of Sequence Variations in Fem A, B and X Genes on the Morphology and Physiology of *S. aureus* Clinical Isolates

Rosy Chikkala and V Sritharan

Background

Staphylococcus aureus is an important bacterial pathogen. Worldwide, the prevalence of MRSA infection varies from 5% to 69.1%. Increase in frequency of MRSA globally and hence the need for accurate detection, have led the use of molecular methods for rapid confirmation of *S. aureus* and MRSA. Species specific markers like auxiliary genes (*fem*) and thermonuclease (*nuc*) gene along with the methicillin resistance determinant, *mec*A, have been used in different combinations by several investigators worldwide for the identification of MRSA. The scope and main objective of this study is to determine prevalence of MRSA in clinical isolates in a tertiary care hospital, to determine the major mechanism of methicillin resistance and to design a simple molecular protocol to directly process and screen uncultured clinical samples for MRSA (data published earlier). This abstract focuses on the studies involving investigations to understand the reasons for poor reliability of *fem* genes as species markers and some experiments to investigate the drug sensitivity (data published earlier), physiology, and morphology of the cells in *fem* genes sequence variants which we identified during our initial screening of clinical isolates.

Method:

Morphological Studies

The *fem* variants were grown in Mueller Hinton broth for 24 h and was harvested, centrifuged and the supernatant was discarded. Cells were fixed by adding 1 mL of 2.5% glutaraldehyde and incubated overnight at 4°C. The fixed cells were dehydrated by using 10% increments of ethanol in water from 0% to 100% for 10 min at each step. Cells were dropped onto the cover slip and dried using a critical point dryer prior to mounting on a 20 mm aluminum stubs with double sided adhesive tape and sputtered with gold particles before imaging.

Physiological Studies

To evaluate the influence of *fem* gene variations on bacterial growth we measured the growth curves of *S. aureus* clinical isolates. We randomly selected 25 *fem* variants *S. aureus* isolates for the assay. To determine the temperature tolerance, we randomly selected 25 *fem* variants for the assay.

Designing of *fem*A and *fem*B Primers for sequencing

In an attempt to amplify the complete gene of *fem*A and *fem*B we designed new primers using Primer3 software based on sequences deposited in GenBank database. Using Primer3 online tool we designed 1 set of *fem*X flanking primers, 4 sets of primers for *fem*A and 3 sets of primers for *fem*B to amplify the complete sequence

Results

Cells of femA variant S. aureus were almost spherical in shape and smooth cocci and the cells measure approximately 550-670 nm in size similar to the wild S. aureus (approx. 550nm). Some of the femB variant S. aureus cells were much bigger (830nm-950nm) and also some cells appeared non-spherical when viewed under SEM. femX variants of S. aureus were remarkably small, spherical and smooth.

All the *fem* variants showed no significant difference in growth rate when compared with reference isolate. In order to determine whether the *fem* variants are more susceptible to temperature stress than their wild type, the 25 *fem* variants were plated on blood agar to determine their (viability/virulence) hemolytic capacity as a measure of viable bacteria at respective temperatures. *fem* variants did not show significant difference in temperature tolerance and the lysis pattern on blood agar ranging from 37°C to 50°C when compared with the wild type. Apparently, the *fem* variants did not lose viability when stressed at higher temperatures.

A single nucleotide change (SNP) was noticed in the original *fem*X-PCR primer annealing site (3'end) and the rest of the sequence in the gene were completely homologous to reference *fem*X gene. *fem*X variant showed single base pair changes (SNP) at #582, GA**A** to GA**G** which codes for Glutamic acid. Whether this SNP could result in negative *fem*X-PCR is not clear. *fem*A variant showed single base pair changes (SNP), one at #900 GCG to GCA and the other one at #972 CTA to CTT, which are codons for Alanine and Leucine respectively.

The degeneracy in codon usage did not result in any change in these two amino acids in the protein sequence. There was no polymorphism at the original binding sites of *fem*A primers. 5 single base pair changes (SNP) were noticed at #582 from GT**A** to GT**C**, at #612 CT**A** to CT**T**, at # 894 GC**A** to GC**T**, at #921 CC**A** to CC**G** and at # 1218 TT**A** to TT**G** which code for Valine, Leucine, Alanine, Proline and Leucine respectively. The degeneracy in codon usages did not allow any replacement of any amino acid in the FEMB protein sequence.

Discussion

Our first important observation is that *fem* genes undergo random spontaneous polymorphism and therefore may not be the ideal genetic markers for identification of *S. aureus* by PCR. The second major observation was that there are several clinical isolates of *S. aureus* which could not be identified by *fem*-PCR which we call as *fem* variants and this is being reported for the first time from India. We investigated various physiological and morphological characteristics of the *fem* variants to see the impact (if any) of these sequence variations.

Considering the fact that FEM proteins are involved in cell wall synthesis, certain functions associated with cell wall such as morphology and physiological studies were performed. We have demonstrated that the primers and protocols for *S. aureus* identification reported from different countries do not show 100% sensitivity though specificity was not a problem. We have redesigned the primers with modern software (Primer 3) for *fem*A, *fem*B as PCR target and shown that higher sensitivity could be achieved. All the variants tested positive for *fem*A and B when screened with the new sets of *fem*A and B primers.

The amplicons obtained from *fem* A, B and X variants were sequenced which showed 96% to 98% identity and homology with the GenBank *fem* genes sequences. Sequence homology was done using Emboss local Alignment software. These alignments also showed that *fem*A, *fem*B genes showed only random mutations which were degenerate in the respective codons and they did not affect the amino acid sequence in Fem proteins. Our *fem* gene sequences have been deposited in the GenBank with the following accession numbers: *fem*A: Acc.No.MG970609, *fem*B: Acc.No.MG970610 and *fem*X: Acc.No. MG963788.

We have reported for the first time about polymorphism in *fem* A, B and X sequences from India. As it is known that *S. aureus* genome is quite dynamic, undergoes rearrangement, mutations and changes frequently, it may be desirable to choose a stable genetic marker like the thermonuclease gene, *nuc* for species confirmation. We evaluated the *nuc* gene (data published earlier) and found that it had better sensitivity and there are no reports of any polymorphism anywhere in the world. Since it is desirable in a molecular method, not only to rule in the target pathogen but should also rule out other closely related species in the sample.

Name: Rosy Chikkala[1] and V Sritharan[2]

Afiliation: [1]Global Medical Education and Research Foundation, India, [2]Gleneagles Global Hospitals, India, Email: rosylikerose@gmail.com