

The thesis entitled “**Culture independent detection and characterization of *Streptococcus pyogenes* and its transcriptional gene regulation upon subinhibitory exposure to antibiotics**” deals with the use of culture independent approaches for the detection of pharyngitis causative bacteria directly from the throat swabs; molecular characterization and comparison of *S. pyogenes* isolated from different infection cases and the *in vitro* and *in vivo* analysis of *S. pyogenes* challenged with subinhibitory concentrations of antibiotics. The work elements have been divided into five major parts viz.,

1. Culture independent analysis of throat swabs from pharyngitis patients and healthy subjects by Denaturing Gradient Gel Electrophoresis.
2. Culture independent analysis of throat swabs from pharyngitis patients and healthy subjects by Terminal Restriction Fragment Length Polymorphism.
3. Development of multiplex PCR assay for the detection of pharyngitis causing bacteria directly from the throat swabs of pharyngitis patients.
4. Comparative molecular analysis of *Streptococcus pyogenes* isolates from ocular infections, pharyngitis and asymptomatic children in south India.
5. Effect of subinhibitory concentrations of fluoroquinolones on biofilm production by clinical isolates of *Streptococcus pyogenes*.
6. *In vitro* and *in vivo* analysis of genes involved in virulence, biofilm formation and antibiotic resistance in *Streptococcus pyogenes* exposed to subinhibitory concentrations of antibiotics.

Streptococcus pyogenes is one of the most common cause of bacterial pharyngitis in human, which upon left untreated may result in deadly post-infection sequelae such as scarlet fever, rheumatic heart disease and necrotizing fasciitis. Apart from *S. pyogenes*, a number of other bacteria are also involved in the pharyngitis. A greater understanding of the bacteria involved is needed to improve our knowledge as well as prescribing right method to eliminate the causative pathogen. Increasing knowledge in the field of molecular diagnostics opens up new strategies to explore the changes in bacterial community structure between the throats of pharyngitis and healthy subjects. In addition, PCR based methods allow rapid, accurate and simultaneous detection of more than one causative bacterium directly from pharyngitis throat swabs by means of multiplex PCR. It also allows molecular comparison of *S. pyogenes* strains isolated from different infection

cases. Apart from diagnostics and molecular characterization, the present study is focused on the *in vitro* and *in vivo* effects of subinhibitory concentrations of various antibiotics against *S. pyogenes* isolates.

The section “**Culture independent analysis of throat swabs from pharyngitis patients and healthy subjects by Denaturing Gradient Gel Electrophoresis**” deals with the comparative analysis of throat metagenomes of pharyngitis patients and healthy subjects using nucleic acid based culture independent method Denaturing Gradient Gel Electrophoresis (DGGE). In this study, 40 throat metagenomes each from healthy and pharyngitis patients were taken. The 16S rRNA gene of the metagenomes was amplified through two rounds of PCR. In the first round PCR, ~1500 bp 16S rRNA gene was amplified using universal 16S primers. The second round PCR is a nested PCR in which first round PCR amplicons were used as templates and V3 region of 16S rDNA (~250 bp) was amplified using GC-clamped primers. DGGE reference marker for inter-gel comparison of DGGE gels was prepared using *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Haemophilus influenzae*. DGGE banding patterns generated directly from the throat swabs showed considerable difference between healthy and pharyngitis samples. The number of separate bands in the studied samples ranged from 3 to 26 in healthy subjects and 2 to 29 in the case of pharyngitis samples. Comparative band analysis showed the presence of more pathogens in pharyngitis samples than the healthy subjects. *S. pyogenes* in 8 (20%); *S. aureus* in 9 (22.5%); *P. aeruginosa* in 4 (10%); *H. influenzae* and *K. pneumoniae* in 7 (17.5%) in healthy subjects’ throat swabs and *S. pyogenes* in 20 (50%); *S. aureus* in 16 (40%); *P. aeruginosa* in 9 (22.5%); *H. influenzae* in 13 (32.5%) and *K. pneumoniae* in 19 (47.5%) in pharyngitis samples. Sequencing of dominant bands revealed the presence of uncultured *Capnocytophaga* sp., *Enterobacter* sp., *Serratia* sp. and *Aeromonas* sp. in the throat metagenomes.

The section “**Culture independent analysis of throat swabs from pharyngitis patients and healthy subjects by Terminal Restriction Fragment Length Polymorphism**” deals with the comparative analysis of throat metagenomes of pharyngitis patients and healthy subjects using terminal restriction fragment length polymorphism (T-RFLP). For T-RFLP analysis, metagenomic DNA was extracted from 10 throat swabs each from healthy and pharyngitis patients. The 16S rRNA genes of

bacteria present in throat metagenomes were amplified by PCR with 6-carboxy-fluorescein (Fluorophore) - labeled universal forward primer (6-FAM - 27F) and a universal reverse primer (1513R). The 16S rDNAs were digested with restriction enzymes with 4bp recognition sites such as *MspI* or *RsaI* and analyzed by using an automated DNA sequencer. T-RFLP patterns were numerically analyzed using computer programs. From analysis of the throat bacterial community, patterns derived from *MspI* and *RsaI* digested samples of healthy subjects and pharyngitis patients were grouped into different clusters, though *RsaI* digested samples showed some uncertainty. Pharyngitis throats generated an average species richness of 9 (± 2.1 [SD]) and 10 (± 2.9) for *MspI* and *RsaI* digests respectively, whereas healthy throats generated 6.3 (± 1.2) and 6.1 (± 1.5) in *MspI* and *RsaI* digests, respectively. These results suggest that samples from pharyngitis patients contain an unexpected diversity of causative bacteria. The pharyngitis throats were colonized with a rich diversity of bacterial species than that of healthy throats. In T-RFLP analysis, the reference strain, *Streptococcus pyogenes* SF370 was used for T-RF comparison. The T-RF patterns of the reference strain coincides with the *Streptococcus pyogenes* T-RFs generated from the metagenomes.

The section “**Development of multiplex PCR assay for the detection of pharyngitis causing bacteria directly from the throat swabs of pharyngitis patients**” deals with the development of a pentaplex PCR assay for rapid and simultaneous detection of five oral pathogens namely *S. pyogenes*, *S. aureus*, *P. aeruginosa*, *N. gonorrhoeae* and *K. pneumoniae* directly from the throat swabs of pharyngitis patients. The designed species-specific primers detected the five targeted pathogens without any cross reaction. Multiplex PCR amplification of the five species specific genes was achieved by optimizing the PCR conditions. 16S rRNA gene was taken as internal control. Annealing temperature of 58°C for 1 min was found to be the optimum temperature for the pentaplex PCR amplification. The sensitivity checking using serially diluted DNA (20ng to 1.25ng) showed unambiguous amplification up to 2.5ng of DNA and slight amplification at 1.25ng. Metagenomic DNA (n=39) isolated from throat swabs of pharyngitis patients were used to evaluate the developed assay. The multiplex PCR assay revealed the presence of *P. aeruginosa*, *S. aureus* and *S. pyogenes* in 2 throat swab samples each. None of the metagenomes showed the presence of *N. gonorrhoeae* and *K. pneumoniae* or the combination of *P. aeruginosa*, *S. aureus* and *S. pyogenes*. This study

once again proves that multiplex PCR based pathogen detection is an effective tool in the field of clinical diagnostics.

The section “**Comparative molecular analysis of *Streptococcus pyogenes* isolates from ocular infections, pharyngitis and asymptomatic children in south India**” deals with the molecular characterization of *S. pyogenes* isolates from ocular, pharyngitis and asymptomatic carrier cases. In the present study, *emm* type surveillance as well as its association with toxin gene profiles was analyzed. *S. pyogenes* was detected in 20 isolates of the ocular infected cases such as lacrimal abscess, corneal ulcers and mucocoele. For noninvasive isolates, 370 pharyngitis cases and 400 asymptomatic school children were screened and recovered 33 pharyngitis and 14 carrier isolates respectively. Fourteen *emm* type distributions were observed in ocular isolates whereas 11 *emm* types each were noticed in pharyngitis and asymptomatic carrier isolates. The two dominant *emm* types, *emm49* and *emm63* were accounted for 33% of the total *S. pyogenes* isolates. Among ocular isolates, *slo*, *smeZ*, *speB* and *speG* were found in >50% of isolates and in pharyngitis *smeZ* (48%), *speB* (45%) and *speG* (42%) genes were found to be prevalent. Alarmingly, carrier isolates showed more prevalence to virulence genes than the ocular and pharyngitis isolates with *speF* (79%), *speB*, *speG* (64%), *slo* and *sil* (64%). Among the three groups, pharyngitis isolates harbored more *prtF1* (33%) and *prtF2* (94%) genes than the asymptomatic carriers (28% and 71%) and the ocular isolates (45% and 40%). 450 bp size band in *prtF1* and 350 bp size band in *prtF2* showed dominance. Among the three groups tested, the distribution of *ermB* and *mefA* was high in pharyngitis isolates (30%) where 10 isolates showed the presence of both genes. None of the isolates showed the presence of *ermA* and *tetO* genes. Dendrogram generated based on the virulence and antibiotic resistance gene profiles revealed that the isolates are intermingled (except few clusters), irrespective of their *emm* types and isolation source.

The section “**Effect of subinhibitory concentrations of fluoroquinolones on biofilm production by clinical isolates of *Streptococcus pyogenes***” deals with revealing the effects of sub-MICs of fluoroquinolones (FQs) on *S. pyogenes* biofilm formation. In this study, biofilm forming six M serotypes M56, st38, M89, M65, M100 and M74 of *S. pyogenes* strains were challenged against four FQs namely, ciprofloxacin, ofloxacin, levofloxacin and norfloxacin. The antibiofilm potential of these FQs were analyzed at their subinhibitory concentrations (1/2-1/64 MIC) using biofilm assay, XTT reduction

assay, Scanning Electron Microscopy and Confocal Laser Scanning Microscopy. From the four FQs tested, ofloxacin and levofloxacin at 1/2 MIC showed the maximum inhibition (92%) of biofilm formation against M56 and M74 serotypes. FQs effectively interfered in the microcolony formation of *S. pyogenes* strains at 1/2-1/8 sub-MICs. Interestingly, inhibition of biofilm formation was greatly reduced beyond 1/16 MICs and allowed biofilm formation. XTT reduction assay revealed the increase in metabolic activity of *S. pyogenes* biofilm against the decrease in FQs concentration. In addition, SEM and CLSM validated the potential of sub-MICs of FQs against the six *S. pyogenes*. These results confirm that all the four FQs are concentration dependent in inhibiting *S. pyogenes* biofilm formation. FQs at proper dosage can be effective against *S. pyogenes* and lower dosage concentrations may allow the bacteria to form barriers against the antibiotic in the form of biofilm.

Finally, the section “***In vitro* and *in vivo* analysis of genes involved in virulence, biofilm formation and antibiotic resistance in *Streptococcus pyogenes* exposed to subinhibitory concentrations of antibiotics**” deals with the *in vivo* and *in vitro* effects of sub-MICs of antibiotics on biofilm and non-biofilm forming *S. pyogenes* strains. MIC determination by broth microdilution method showed the highest MIC value of 1 µg/ml for SP7 and SF370 isolates against ofloxacin. SF370 showed the lowest MIC value of 0.03 µg/ml against erythromycin. Sub-MICs of 1/2 to 1/64 MICs were utilized to challenge *S. pyogenes* strains. Sub-MICs treated *S. pyogenes* were assessed for autoaggregation ability and found that it varies between the strains. In biofilm forming strains SP7 and SF370, the average autoaggregation was observed to be 0.25 and 0.7nm. Whereas, the non-biofilm former SP24 showed an average of 0.03nm of autoaggregation. mRNA isolated from the sub-MIC treated bacterial cells were utilized for analyzing the virulence, biofilm and antibiotic resistance associated genes. Real time PCR analysis showed gene regulation in concentration dependent manner. The analysis showed increase in expression at the higher and lowest sub-MICs and showed a decrease in gene expression at the middle sub-MICs. These results have correlation with the autoaggregation assay results where, most of the antibiotics showed autoaggregation in concentration dependent manner. *In vivo* analyses using *C. elegans* through nematode liquid killing assay shows biofilm forming strains SP7 and SF370 extending its survival up to six days, whereas complete mortality occurred within four days in the case of non

biofilm former SP24. In addition, lower sub-MICs such as 1/8 to 1/64 MICs showed lethal effects within 48h whereas the control showed less lethal effect. The results of the present study substantiate that the lower sub-MICs of antibiotics enhance the virulence of *S. pyogenes*. Hence, from the present study, it is evident that usage of antibiotics at proper concentration is very crucial to protect the mankind from life threatening infections.