CHAPTER 4

NON SPECIFIC AMPLIFICATION OF HUMAN DNA BY

Streptococcus pneumoniae Lyt A PRIMER

4.1 INTRODUCTION

With the discovery of polymerase chain reaction (PCR) (Gray and Fedorko 1992) nucleic acid based amplification tests (NAATs) are being extensively used in many diagnostic laboratories across the world (Buchan and Ledeboer 2014). Their short turn-around time, high sensitivity and specificity make them ideal tests for identification of uncultivable, unidentifiable and unknown pathogenic microbes from clinical samples (Ruskova and Raclavsky 2011). Several modifications of NAATs are available at present, some of which have become considerably cheaper due to substantial increase in clientele base (Maurer et al. 2017). Among them, quantitative or real-time PCR (Q-PCR) is now the most widely used test and is being used extensively for molecular detection of pathogenic microbes from clinical samples (CDC 2011). Although cost can be a limiting factor, many laboratories use Q-PCR for rapid diagnosis of life-threatening clinical conditions. In recent times, multiplex PCR have been used on a real-time PCR platform for the simultaneous detection of multiple agents, any of which may cause the same clinical condition. Many commercial kits are available in the market for Q-PCR testing, but their use is often constrained by cost and availability. One way to circumvent this problem is to standardize in-house techniques which when properly standardized and evaluated, can be economically viable (Burd et al. 2010).
Laboratory developed in house PCR tests are therefore meant to reduce cost without compromising on their sensitivity and specificity. Optimisation of such tests requires standardization of equipment, reagents and interpretation. Introducing an appropriate internal control (IC) will help to determine if the amplification is carried out properly or if it is inhibited by non-specific inhibitors (Burd 2010). Another parameter that requires stringent validation is the primers used in the testing (OIE 2008). This starts with the delineating various test characteristics of the primers including analytical specificity, sensitivity and assay reproducibility (Burd 2010). Normally, the specificity of the chosen primers is determined by testing them against genetically related and unrelated microbial pathogens as well as clinical material obtained from humans with diseases that may mimic the intended target for which the assay is being designed (OIE 2008). In general, primers that show such cross-reaction or non-specific amplification are not chosen for designing PCR protocol; otherwise strict optimisation should be implemented for those primers in the validation phase. In our early experiments with the standardisation of HRMA for the molecular detection of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis*; *SP LytA* primer chosen for *S. pneumoniae*, selected from a CDC protocol showed non-specific amplification with human DNA in wet laboratory experiments. This chapter describes the details of these observations and demonstrates how the problem was solved to differentiate the melt curve of *SP* DNA from the human DNA amplicon.

### 4.2 MATERIALS AND METHODS

HRMA was standardized using DNA extracted from *Streptococcus pneumoniae* (ATCC strain R 49619TM) obtained from the Division of Bacteriology, Microbiological Laboratory, Coimbatore, India using Type-
HRM™ PCR kit (Qiagen, Hilden, Germany) in triplicates in Rotor Gene Q Thermal Cycler. The test was done according to the manufacturer’s instructions. The primers used were SP Lyt A F373 (ACGCAATCTAGCATGAAGCA) and SP Lyt A R424 (TCGTGCTTTTTAATTCCAGCT) and were obtained from Integrated DNA Technologies, Singapore. The program for HRMA was followed as set in the software while the Ct values, end point fluorescence level and melting profile were assessed by HRMA Software.

Melt or dissociation curve was generated after the completion of PCR by raising temperature from 65°C to 99°C with 0.1°C incremental order for 2s each ramp rate in Rotor Gene Q5plex Real Time PCR machine with HRM capability. The normalized raw data by selecting linear regions before and after the melting transition and the derivative plot were generated by inbuilt HRMA software in the same Rotor gene Q 5plex HRMA machine. To optimize the HRMA profile, the derived melt curve was assessed to check the purity of the product and primer dimer formation (Nan 2011).

4.2.1 Specificity and sensitivity checking in dry and wet experiments

The annealing temperature, expected amplicon size and the predicted melt temperature (Tm) of the amplicon were calculated through Bioinformatics tools of NEB Tm calculator (version 1.9.4; New England Biolabs Inc. [https://www.neb.com/]), primer BLAST database (www.ncbi.nlm.nih.gov/tools/primer-blast) and a free online Oligo Calc (version 2; Northwestern University, Chicago, IL [www.simgene.com]).

The cross reactivity of the primers was checked against nucleotide database of reference sequences from representative genomes of
seven species of *Streptococcus*, 14 unrelated pathogenic bacteria, nine pathogenic viruses, three fungal pathogens, two protozoal pathogens as well as human genome as an adjunct to wet lab experiments through NCBI primer-BLAST Search Analysis [www.ncbi.nlm.nih.gov/tools/primer-blast/].

The specificity of the primers was also determined in wet lab experiments against the same bacterial, viral, fungal and protozoal pathogens listed above. For this, DNA was extracted from them and subjected to HRMA as described earlier. In another experiment, HRMA was also performed on DNA extracted from human clinical material that included CSF, blood/plasma, tissue, BAL fluid and bronchial wash which were negative for *SP* by Gram stain / culture and which may mimic the intended target for which the assay was being designed.

In another wet lab experiment, sensitivity was determined in a spiked experiment by calculating the lower limit of detection threshold (LLT). For this, CSF samples negative for *SP* DNA were pooled and used as a single sample. A known concentration of ATCC *Streptococcus pneumoniae* DNA (determined as $2.53 \times 10^5$ copies/μL using Qubit fluorometer), a series of ten-fold dilutions starting from $10^{-1}$ (25300 copies/μL) through $10^{-7}$ (0 copy/μL) were made in TE buffer. From each dilution, 1μL was taken and spiked in to 200μL of pooled CSF sample and subjected to extraction using Bioneer Accuprep genomic DNA extraction kit followed by amplification in Rotor gene Q. The last dilution giving amplification was taken as the end point. The LLT values were confirmed by repeating the testing of these dilutions in triplicates.

To further confirm the cross reactivity of the primers, three CSF samples reported positive for high, medium and low concentrations of *SP*
DNA by a commercial kit, the FTD (Fast Track Diagnostic kit, IVD, Luxemburg, Germany) were subjected to HRMA and Tm value determinations. The results were compared with the amplification curve obtained by the FTD kit.

4.2.2 Confirmation of amplicon specificity by sequencing

The amplified product obtained from the ATCC *Streptococcus pneumoniae* strain and known positive CSF were sent to Eurofins genomic Pvt Ltd, Bangalore for sequencing to confirm the sequence of the primers. All sequencing reports were analysed through Gene Bank.

4.3. RESULT AND DISCUSSION

4.3.1. Analysis of *SP LytA* primer

The dry lab experiments showed that the annealing temperature, expected fragment length and the predicted Tm of *SP Lyt A* primer to be 53°C, 75 bps and 75°C respectively. Wet lab experiments showed that the amplification of *SP* DNA started at the 17th Ct cycle and had a distinct Tm of 77.78°C (Figure 4.1). The average Tm value based on three repeat testing was 77.3°C with a range of 77°C –78°C. It showed a difference of +2°C to 3°C with the dry lab data.
Figure 4.1. The amplification curve and Tm value of ATCC *Streptococcus pneumoniae* strain in the wet lab experiment

4.3.2 Analytical specificity of *SP LytA* primer in dry lab

Testing of *Lyt A* primer with seven species of *Streptococcus*, 14 unrelated pathogenic bacteria, nine pathogenic viruses, three fungal pathogens and two protozoal pathogens did not show any cross reactivity in dry lab experiments (Table 4.1). However, BLAST analysis showed cross reaction with human chromosome 1, 7 and 19 (Figure 4.2).

Table 4.1. List of microbial species whose genomes were tested to check cross-reactivity of *SP LytA* primer in dry lab experiments

<table>
<thead>
<tr>
<th>Representative Genomes</th>
<th>Microbial Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcal sp.</em></td>
<td><em>S. dysgalactiae, S. agalactiae, S. anginosus, S. constellatus, S. intermedius, S. sanguinus</em></td>
</tr>
<tr>
<td>Pathogenic viruses</td>
<td><em>HSV-1, HSV-2, Varicella-Zoster virus, EBV, CMV, HSV-6A Enterovirus, Measles virus, Japanese encephalitis virus</em></td>
</tr>
<tr>
<td>Fungal Pathogens</td>
<td><em>Candida sp. Cryptococcus neoformans, Aspergillus sp.</em></td>
</tr>
<tr>
<td>Protozoal Pathogens</td>
<td><em>Entamoeba histolytica, Plasmodium falciparum</em></td>
</tr>
</tbody>
</table>

103
<table>
<thead>
<tr>
<th>Specificity of primers</th>
<th>Primer pair</th>
<th>Sequence (5'-&gt;3')</th>
<th>Length</th>
<th>Tm</th>
<th>GC%</th>
<th>Self complementarity</th>
<th>Self 3' complementarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>ACGCAATCTAGCAGATGAAGCA</td>
<td>22</td>
<td>60.42</td>
<td>45.45</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Reverse primer</td>
<td>TCGTGCGTTTTAATTCCAGCT</td>
<td>21</td>
<td>58.85</td>
<td>42.86</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

**Products on intended target**

> **AC080080.5** Homo sapiens BAC clone RP11-511H23 from 7, complete sequence  
product length = 3484  
Reverse primer 1 TCGTGCGTTTTAATTCCAGCT 21  
Template 91290......CA...T........C 91270  
Reverse primer 1 TCGTGCGTTTTAATTCCAGCT 21  
Template 87807 CT...T.........G.... 87827

> **NG_047068.1** Homo sapiens protein phosphatase 2 scaffold subunit Aalpha (PPP2R1A), RefSeqGene on chromosome 19  
product length = 979  
Reverse primer 1 TCGTGCGTTTTAATTCCAGCT 21  
Template 4313..T........C..G.....A 4293  
Reverse primer 1 TCGTGCGTTTTAATTCCAGCT 21  
Template 3335..A...C.G.A....... 3355

> **AC010320.10** Homo sapiens chromosome 19 clone CTD-252513, complete sequence  
product length = 979  
Reverse primer 1 TCGTGCGTTTTAATTCCAGCT 21  
Template 32541..T........C..G.....A 32521  
Reverse primer 1 TCGTGCGTTTTAATTCCAGCT 21  
Template 31563..A...C.G.A....... 31583

> **AL139128.24** Human DNA sequence from clone RP11-101O6 on chromosome 1, complete sequence  
product length = 1139  
Reverse primer 1 TCGTGCGTTTTAATTCCAGCT 21  
Template 52633ATC.TT................. 52613  
Reverse primer 1 TCGTGCGTTTTAATTCCAGCT 21  
Template 51495G.AC..C.G........... 51515

**Figure 4.2.** The BLAST analysis data showing cross reaction of *SP Lyt A* primer with human chromosome 1, 7 and 19
4.3.3. Wet lab experiments for specificity of the primers

Amplification was observed with DNA extracted from the ATCC *Streptococcus pneumoniae* strain alone and not with any of the related streptococcal species or unrelated bacterial, viral, fungal and protozoal pathogens (Data not shown). This is in accordance with the results of the dry lab experiments.

4.3.4 Analytical sensitivity of SP Lyt A primer and its cross reaction with human chromosome

Figure 4.3 shows the melt curve and Tm values of the seven ten-fold dilutions in the spiking experiment. The set of larger peaks seen between 75°C and 80°C belonged to the SP DNA. The smaller peaks seen later in the Ct cycles had a peak temperature 80 ± 0.1-0.9°C and seem to indicate some non-specific cross reaction. Since dry lab data indicated cross reactivity with human chromosome, a cross reactivity with human DNA was considered.

![Figure 4.3. The melt graph of SP Lyt A primer in spiking experiment. Cross reactivity of SP primer started only after 30th Ct amplification with the Tm 80.96°C using CSF as sample matrix](image)
Table 4.2 shows the Ct and Tm values of the ATCC *Streptococcus pneumoniae* spiking experiment done in ten-fold dilutions in triplicates. The average Tm values of amplicons in dilutions $10^{-1}$ through $10^{-4}$ varied from $76.56^0{\text{C}}$ to $76.70^0{\text{C}}$ which corresponded to Ct cycles of 20.43 to 29.81. In contrast Tm values for dilutions $10^{-5}$ and $10^{-6}$ were $>80^0{\text{C}}$ at Ct cycles of $>30$. Thus presence of an amplicon with a higher Tm value and Ct cycle confirmed the existence of a second amplicon.

**Table 4.2. Ct cycle and Tm values of different dilutions of target DNA in the spiked experiments after amplification**

<table>
<thead>
<tr>
<th>Dilutions of the target DNA*</th>
<th>Average Ct cycle</th>
<th>Average Tm value ($^0{\text{C}}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$</td>
<td>20.43</td>
<td>76.69</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>24.34</td>
<td>76.56</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>26.25</td>
<td>76.64</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>29.81</td>
<td>76.70</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>31.99</td>
<td>81.01</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>32.06</td>
<td>80.91</td>
</tr>
</tbody>
</table>

*Each dilution was done in triplicates. The values for Ct cycles and Tm are the average for each dilution*

Table 4.3 shows the results of the unspiked experiments on human clinical specimens. Amplification was noted at Ct cycle of 30 or later while the Tm values varied from 81.53 to 82.90°C with an average of 82.03°C. This coincided well with the results of the spiked experiment where the nonspecific cross reaction was observed beyond 30th Ct with an average Tm value of 80.96°C. This confirms that *SP Lyt A* primer have some non-specific cross reactivity at higher dilution and beyond a Ct of 30.
Table 4.3. Ct and Tm values of DNA extracted from human clinical samples without SP DNA

<table>
<thead>
<tr>
<th>Clinical sample</th>
<th>Ct</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>32</td>
<td>81.95</td>
</tr>
<tr>
<td>Blood</td>
<td>30</td>
<td>82.9</td>
</tr>
<tr>
<td>Plasma</td>
<td>32</td>
<td>82.47</td>
</tr>
<tr>
<td>BAL</td>
<td>31</td>
<td>81.53</td>
</tr>
<tr>
<td>Bronchial wash</td>
<td>31</td>
<td>81.73</td>
</tr>
<tr>
<td>Tissue</td>
<td>30</td>
<td>81.62</td>
</tr>
</tbody>
</table>

4.3.5 Sequencing of ATCC *Streptococcus pneumoniae* and human genome amplicons

The reliability of the amplified product was confirmed by sequencing. The amplified product of *SP* ATCC DNA sent for sequencing failed due to limitations in conventional sequencing. For obtaining good sequencing data, the quality & specificity of the primer and the complexity & purity of the template DNA is critical parameter. Since quality score of the bases in chromatogram will be less due to the poor initial resolution, sequencing of samples below 100bp length is critical and the pre-sequencing gel QC failed for amplicons of below 100bp in length. Hence, the *SP* amplicon which had 75bps were not qualifying for sequencing (Figure 4.4. (a). Thus, sequencing showed some kinds of limitations in identifying bases. However, HRMA found those nucleotide sequences exactly by revealing the standard Tm of the *SP* Lyt A amplicon as 77.78°C and was also matched with the results of conventional gold standards i.e. Gram stain & culture and also by other IVD approved molecular testing.
Figure 4.4. a) Gel image of SP ATCC DNA not qualified for sequencing due to less quality score of the bases in chromatogram lead to initial poor resolution. b) Ladder specifications. c) Amplicon of known SP positive CSF in gel image

Since the wet lab experiments with spiked samples showed two peaks, it was decided, to check the specificity of SP primer against Homosapiens DNA and the amplified product of known SP positive CSF specimen as a source of human DNA sent for sequencing. Pre sequencing gel showed dispersed bands of different intensity revealing poor quality of SP amplicon (Figure 4.4 (c); confirming the suspicion of SP Lyt A primer cross reactivity with Homosapiens DNA as analyzed by insilico dry runs revealed that SP Lyt A primer cross reacted with human DNA chromosome No: 1, 7, and 19 (Table 4.2).

Finally, sequencing revealed that the amplified product of Known SP Positive (by conventional culture, Gram Stain and HRMA) CSF Specimen containing the sequences of Human DNA and found that 13 bps from forward
and 10 bps from reverse primers of \textit{SP Lyt A} gene cross reacted with human DNA (Figure 4.5 a and b). The sequencing result was subjected to BLAST analysis and this showed that the amplicon contained sequences of human chromosome 20 and 20q12. This confirmed that the \textit{SP} primer \textit{Lyt A} showed cross reactivity with human DNA. The sequence was found to be associated with Homo sapiens protein tyrosine phosphatase, receptor type T (PTPRT) gene on chromosome 20 (Figure 4.6). Since \textit{SP} sequencing showed cross reactivity with human DNA, the specificity of \textit{SP} primer was in question for the detection of \textit{SP} amplicon which is below 100 bp in size along with the sequences of human DNA. But still, it was distinguished in HRMA by checking the Tm of \textit{SP} DNA as 77.7°C and Human DNA as 81.2°C.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_5.png}
\caption{a). \textit{SP LytA} primer cross reactions with \textit{Homo sapiens} protein tyrosine phosphatase, receptor type T (PTPRT), ref seq gene on chromosome 20 (Sequence ID: NG_033880.1) \hfill b). Amplification of \textit{SP Strain 122} autolysin gene by \textit{SP} primer \textit{Lyt A} (GenBank: KY581565.1)}
\end{figure}
Figure 4.6. Nucleotide blast reports of sequenced nucleotides of known positive SP amplicon
In second confirmation, the known reference sequence of human genome (Human Genomic DNA Ref No: 900421(Affymetrix); Lot No – 4043072, 2016-01-27) was checked with HRMA and found that the amplification occurred and generated a single dominant melt peak at 81.95°C (Figure 4.7).

![Graph showing SP Primer Cross Reactivity with Human Genomic DNA Ref No: 900421(Affymetrix)]

**Figure 4.7. SP Lyt A primer cross reactivity with Human Genomic DNA Ref No: 900421(Affymetrix)**

### 4.3.6. Comparison of HRMA with a commercial kit (Fast Track Diagnostics Multiplex PCR Kit, IVD, Germany)

The non-specific amplification was further confirmed by doing HRMA on three CSF samples reported positive for high, medium and low concentrations of SP DNA by a commercial kit (Fast Track Diagnostic kit, IVD, Luxemburg, Germany). The results showed that Ct values for the three samples were 10.30, 22.77 and 27.17 and Tm values were 78.55°C, 78.70°C and 78.90°C respectively (Figure 4.8). The amplification curves in the high and medium positives were steep indicating relatively high concentrations of SP DNA while the curve in the low positive was relatively poorly formed. The low positive sample showed a peak starting at ct cycle 27.17 with Tm values of 78.9°C and
83.3°C for SP and human DNA respectively. More interestingly, the low positive alone showed two peaks, the second one at Ct cycle 27.17 and with Tm value 83.3°C represented the human genome.

<table>
<thead>
<tr>
<th>PCR HRMA (Type iT HRM™ Kit, Qiagen, Germany)</th>
<th>Fast Track Diagnostics (FTD) IVD Approved molecular diagnostics kit, Luxembourg, Germany</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amplification Graph</strong></td>
<td><strong>Melt plot</strong></td>
</tr>
<tr>
<td><em>SP high level</em> POSITIVE</td>
<td><em>SP high level</em> POSITIVE</td>
</tr>
<tr>
<td>Ct: 10</td>
<td><em>SP Tm - 78.55</em></td>
</tr>
<tr>
<td><em>SP medium level</em> POSITIVE</td>
<td><em>SP medium level</em> POSITIVE</td>
</tr>
<tr>
<td>Ct: 22</td>
<td><em>SP Tm - 78.70</em></td>
</tr>
<tr>
<td><em>SP Low Level</em> POSITIVE</td>
<td><em>SP Low level</em> POSITIVE</td>
</tr>
<tr>
<td>Ct: 27</td>
<td><em>SP Tm - 78.90</em> Human DNA Tm -83.30</td>
</tr>
<tr>
<td><em>SP NEGATIVE</em></td>
<td><em>SP NEGATIVE</em></td>
</tr>
<tr>
<td>Ct: 0</td>
<td><em>SP Tm-0</em></td>
</tr>
</tbody>
</table>

Figure 4.8. High Resolution Melt Analysis of CSF samples positive for high, medium and low level SP DNA by Fast Track Diagnostic Kit
In general, validation of intended primers for the identification of infectious etiology purely depends on the stringent specificity checking using clinical specimens (Burd 2010 and OIE 2008). Incidentally, the primers selected from the published articles are more specific to the respective targets but showing little cross reactivity with human DNA. This was proved by both dry and wet runs in MBL. One such case attempted for the diagnosis of bacterial meningitis is *Streptococcus pneumoniae* (*SP*) *Lyt A* primer adapted from Centre for Disease Control (CDC 2011). Though the primers are more specific to autolysin gene of *SP*, little cross reactivity has been observed with Homosapiens DNA leads to the generation of short length amplicons while doing PCR in metagenomics. Moreover, detection through high resolution melt analysis showed non-specific melt at different juncture. Continuous validations of this primer overcome the issues related to specificity and finally it was distinguished by two distinct melt; SP DNA at 77±2°C and human DNA at 81.8±1.5°C.

*In silico* analysis of *SP Lyt A* primer giving a rough idea of predicting its annealing temperature, expected amplicon length and the predicted melting temperature. Based on this prediction, HRMA was started to standardize the meningitis panel using commercial RT PCR kit capable of performing High Resolution Melt Analysis (Type it HRM Kit, Qiagen, Germany) in Rotor Gene Q real-time cyclers. No optimization of the Mg2+ concentration or the annealing temperature is required while using this kit except to stick on the optimized protocol in the handbook. Moreover, *SP Lyt A* primer cross reacted with human chromosome No: 1, 7, and 19 thereby generating the product lengths of 1139, 3484 and 979 respectively as per dry lab data (Figure 4.2). However, the dry lab data not matched with wet experiment generating 171 bp non specific amplified products of human chromosome no 20 and 20q12. This variation is mainly due to the PCR protocol
set up used in both dry and wet experiments including annealing temperature, amplification seconds and cycles etc.,

In house standardization of SP primer with Type it HRM kit using ATCC strain of *Streptococcus pneumoniae* showed the amplification Ct (17) and the derivative melt plot at Tm – 77.78°C with the fluorescent intensity showing the height of melt peak at 2.8 df/dt ultimately revealing the concentration of DNA in the amplified product. With this optimization of predicting the Tm of SP as 77.78°C, the clinical CSF specimens generally declared as positive for SP by gram stain and culture was tested to detect the SP DNA by HRMA; thereby checking the same Tm of SP as 77.78°C in CSF specimen as well. It was obtained the Tm as 77.5°C. Continuous repetition showed the Tm range as 76 –79°C for SP DNA at different concentrations in clinical CSF specimens.

Upon validation especially in the stage of spiking experiment, higher dilution showed an anonymous peak at 80.96 ± 0.5°C triggered a suspicion on the specificity of SP primer while using clinical specimens. In an another experiment using different clinical specimens declared as negative for SP by other molecular tests were tested for HRMA using the same SP Lyt A primer. It also generated the non-specific cross reactive melt peak at 81.8±0.5°C thereby confirming the non-specific products as seen in the sample matrix of CSF specimen. In order to confirm the non-specific products, sequencing was done for the known SP positive CSF specimen to ratify the presence of both SP and human DNA in the same amplicon. Unfortunately, sequencing was supporting for the presence of human DNA in the sent amplicon not for the SP DNA. As pre sequencing gel showed dispersed bands of different intensity revealing poor quality of SP amplicon as mentioned earlier, thereby confirming the suspicion
of more than one product while using *SP Lyt A* primer in clinical specimens. Finally, sequencing revealed that the non specific cross reactive amplified products belongs to human DNA chromosome 20 and 20q12.

It was decided to send the pure amplicon derived from the *SP ATCC* strain in order to prove *SP Lyt A* primer detects *Streptococcus pneumoniae* autolysin gene as well. Unluckily, the amplified product of *SP ATCC* DNA sent for sequencing failed due to limitations in conventional sequencing. For obtaining good sequencing data, the quality and specificity of the primer and the complexity and purity of the template DNA is critical parameter. Since quality score of the bases in chromatogram was less due to the poor initial resolution, sequencing of samples below 100bp length is critical and the pre-sequencing gel QC failed for amplicons of below 100bp in length. Hence, the *SP* amplicon which has 75bp not qualifying for sequencing as mentioned in results section. Thus, sequencing showed some kinds of limitations in identifying bases.

Finally, it was suggested to compare the known *SP* positive detected by other IVD approved molecular kits with HRMA. One such kit used was Fast Track Diagnostic kit, Luxembourg to compare the high, medium & low level positives of *SP* detected by FTD kit with Type it HRM kit using *SP Lyt A* primer. All the levels were detected in both the kits. As found earlier in spike experiment, the low level positive showed two melt peak at 78 and 83 as well saying that in higher dilution the proportion of amplified human DNA increased as the concentration of *SP* DNA decreased and matched with the results of IVD approved FTD molecular kits. However, *SP* sequencing failed to prove the specificity of *SP* primer for the detection of *SP* amplicon which is below 100 bp in size along with the sequences of human DNA in clinical
specimens. But still, it was distinguished by checking the Tm of *SP* DNA as 77±2°C and Human DNA as 81.8±1.5°C by HRMA technique.

It was concluded that choosing primers from the published articles using real time probe based RT PCRs generated cumbersome results while practicing intercalation based RT PCR with melt curve analysis. Instead of that, primers already validated by conventional PCR are more prompt for optimizing intercalation based real time PCR-HRMA; since it was validated by the published authors and proved not to generate primer dimers and non-specific amplification using gel documentation. While intercalation based RT PCR are more vulnerable to generate nonspecific amplification and primer dimer formations, the strict primer specificity is mandatory for the detection of indented targets in biological specimens. But in case of probe based assays, though the primers are little nonspecific, the probes are more specific which binds to the mid of amplicon if it is specific; otherwise, it will not bind; thereby providing dual sensitivity not to miss the intended target anywhere. The primers taken from the article based on probe and it didn’t miss any of the *SP* positive CSF specimens even though the primers showed little cross reactivity with human DNA generating short length amplicon from chromosome No; 20 as proved by sequencing. Rather than seeing primer specificity stringently, amplicon specificity plays an important role in real time PCR based on probes (Lenka Ruskovaa 2011). Since labeled probes are expensive to design a real time PCR set up in cost effective manner, validation by stringent primer specificity followed by intercalation based detection using novel saturated third generation dye such as Eva Green in low cost PCR mixes i.e., Type it HRM kit (Qiagen, Germany) serves as a very good adjunct to probe based real time PCR assays. Human DNA cross reactivity has been well documented by many researchers including Faria et al. (2015) against 16SrRNA primer explaining that the proportion of amplified human DNA increased as the concentration of
bacteria decreased. The present study also experienced the same fact while doing spiking experiment using *SP Lyt A* primer for the detection of *Streptococcus pneumoniae* in CSF specimen.

This study describes the cross reaction of *SP LytA* primer used in the PCR for HRMA with a human chromosome. This observation was made when spiking experiments with ATCC *Streptococcus pneumoniae* strain and unspiked clinical samples were carried out to determine the analytical specificity of the primers. Sequencing of the amplicon obtained in the spiking experiment and subsequent BLAST analysis showed the cross reaction was with the human chromosome number 20. Dry lab experiments had shown cross reaction with human chromosome 1, 7 and 19; nevertheless, the result proves the existence of such cross reactions between totally unrelated genomes. Our results demonstrate the need to check for such cross reactions in microbial primers that are used in amplification studies. This should be done initially by dry lab analysis which if found positive should be confirmed with wet experiments.

The initial suspicion of a cross reaction arose when the spiking experiments were conducted to determine the analytical sensitivity of the *SP Lyt A* primers. Amplification of the low positive CSF for *SP* showed two peaks, one before and another one after the 30th Ct cycle. In experiments set up with ten-fold dilutions in triplicates, dilutions up to 10^-4 (up to 29.8 Ct cycle) gave a Tm value of 76.7°C while dilutions from 10^-5 (Ct cycles of 31 – 32) gave a Tm value of 81°C. Thus two amplification peaks were observed with different Tm values at different Ct cycles. Subsequently an unspiked experiment was done with clinical specimens that were negative for *SP* using the *SP Lyt A* primers. All six different types of clinical specimens tested gave amplification curves at
Ct cycle of 31 and with Tm value of 82°C. These results confirmed the earlier results with the spiked samples thereby establishing a cross reactivity between SP Lyt A primer and an unidentified material of human origin. The amplified product was sent for sequencing and the data obtained were analyzed through BLAST analysis. The results showed that the cross reactivity was due to a gene in human chromosome 20 that was identified as *Homo sapiens* protein tyrosine phosphatase R type T. It is important to remember that the primer sequence was taken from a CDC protocol and in the natural course of events one would not have thought of such a cross reaction. But development of amplification peak with different Tm values made us suspect that there may be some problems with the primer sequences. This was found to be a specific cross reaction with a genetic locus of human chromosome number 20.

To confirm nature of the cross reaction, HRMA was done on three CSF samples that yielded high, medium and low SP DNA load with a commercial kit (Figure 4.8). HRMA done on these samples also gave high, medium and low amplification curves; however, in the melt plot, only the low positive sample gave two peaks indicating that cross reaction was observed only when the SP DNA load is relatively low. A possible explanation is that when DNA load is high, the master mix is used up for much of the amplification with very little left for the later Ct cycles of amplification. However little master mix is used up when the load is low so that much of it is available for the amplification during the later Ct cycles. In clinical setting, such cross reactions of the primers may not matter because of the high DNA load in CSF samples of patients with acute pyogenic meningitis. However these observations may vary and therefore it is important to check for any possible primer cross reactivity while standardizing them for the PCR / HRMA protocol.
The present report is the first case of a cross reaction between a specific microbial primer and human genome. In an earlier report, Faria et al. 2015 while standardizing a profiling strategy to identify polymicrobial bacterial DNA in whole blood by 16S rRNA had alluded to sequences that cross reacted with human DNA. Like the results of this study, they also had referred to the low ratio of bacterial to host DNA as a major factor for such a cross reaction. Problem of cross reactivity was solved by deleting the cross reacting sequences from the primer. Such non-specific amplification of human DNA with universal 16S rRNA is well documented (Ozbak et al. 2012; Tong et al. 2012; Edwards et.al.1989; Bosshard et al. 2002; De Vlaminck et al. 2013; Kommedal et al. 2012; Maitra et al. 2015; Yadav et al. 2018 and Huys et al. 2008).

In contrast, the present study observed cross reaction between a specific primer and human genome used for the molecular detection of SP in CSF samples. Clinical samples negative for SP may still contain pus cells and can give a false positive result because it contains human genetic material. This is confirmed by our experiments with unspiked clinical samples that were negative for SP, yet gave amplification at a higher Ct cycle and with a higher Tm value. Our strategy to overcome the cross reaction was to stop the amplification at the 30\textsuperscript{th} Ct cycle beyond which true amplification will not occur while any amplification beyond this will indicate a non-specific amplification. The present finding on the non-specific amplification may explain the high incidence of false positive reactions being reported with Streptococcus pneumoniae in certain automated molecular diagnostic panels that may not use specific probes (Hanson 2016; Leber et al. 2016 and Hanson et al.2016). Such non-specific amplification will not occur in the classical real-time PCR system because the latter makes use of specific probes. Concluded that one must exclude non-specific amplifications while selecting primers for in house standardization of HRMA so that false positive reactions do not interfere
with the target identification in clinical samples. The results show that primers need to be checked for and cleared of such cross reactions before using them for standardization of in-house amplification based tests.

### 4.4. CONCLUSION

Microbial primers used in nucleic acid amplification tests are known to cross react with unrelated and related microbial species as well as human genome. It is therefore important to analyse the primer sequences through bioinformatic and wet lab experiments. In those PCR reactions where a specific probe is used to identify the final amplicon, no such cross reactions are observed with the primers. However, HRMA is done without a specific probe and therefore non-specific amplifications can occur, even with human genomes. Therefore corrective measures should be taken to identify such cross-reactions which can result in non-specific amplification before standardizing HRMA. This study has shown that cross reactions occur only when target DNA is in lower concentrations when compared to contaminating DNA. However these observations may vary and therefore it is better to check for these cross reactions and remove them before standardizing tests with such primers.