CHAPTER 7

SUMMARY AND CONCLUSION

A HRMA technique was standardized for *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis* causing acute pyogenic meningitis, *Mycobacterium tuberculosis* causing TBM and five most common viral agents causing meningoencephalitis in India (Figure 7.1).

![Melt curve patterns and Tm values of 9 microbial species with 10 primers](image)

**Figure 7.1. Melt curve patterns and Tm values of 9 microbial species with 10 primers**

Melt curves of Meningoencephalitis Panel by real-time PCR-HRMA. The *SP* amplicon has a melting temperature around 77.3°C, *HI* -77.7°C, *NM Sod C* - 78.6°C & *NM Ctr A* - 80.3°C respectively. Similarly, *EV* - 84.9°C, *HSV 1*-83.4°C, *HSV 2* - 86.4°C, *VZV* - 76.8°C, *CMV* – 84.5°C, *MTB* - 85.0°C & Pan Bacteria – 82.9°C respectively. *SP*: *Streptococcus pneumoniae*; *HI*: *Haemophilus influenzae*; *NM*: *Neisseria meningitidis*, *MTB*: *Mycobacterium tuberculosis*, *EV*: Enterovirus, *HSV 1/2*: Herpes Simplex Virus 1 and 2, *VZV*: Varicella Zoster Virus, *CMV*: Cytomegalovirus
Testing of the primers for detection of these 9 agents against diverse related and unrelated microbial DNA showed that, by and large, the primers are specific for the agents against whom the primers are intended. However non-specific amplifications due to non-specific cross reactions are found to with related and unrelated species as well as human genome. *SP LytA* primer used for the detection of *SP* in CSF was found to cross react with certain sequences of human chromosome number 20. The genetic locus was identified as *Homo sapiens* protein tyrosine phosphatase R type T. To confirm this, three CSF samples reported as high, medium and low positive by a molecular diagnostic kit were tested by HRMA using the *SP LytA* primer. It was found that only the cross reaction was observed that only in the low positive control. Probably this is due to the availability of sufficient master mix, much of which is still available due to the low yield of bacterial DNA. On the contrary, when the DNA load is high, much of the master mix is used up so that enough of the latter is not available for the primer to cross react with. These results confirm the need for checking for cross reactions and trouble shooting them before validating the primers.

Determination of analytical sensitivity showed that the test could detect 1 copy of DNA molecule / μL at dilutions of $10^{-5}$ to $10^{-6}$ confirming high sensitivity of the test in picking up minute amounts of DNA molecules in CSF samples. Robustness of the test and absence of non-specific inhibition of PCR was confirmed by the inclusion of an internal control which showed amplification during HRMA testing. The coefficient of variation observed in the inter and intra assay was less than 1%; this showed the excellent robustness and portability of this HRMA technique. The melt curves of the four primers were easily distinguishable from one another. The Tm values of *SP LytA*, *HI hpd*, *NM sod C* and *NM ctr A* primers were 77.3°C, 77.7°C, 78.6°C and 80.3°C respectively. Use of two *NM* primers, *sod C* and *ctr A* in a biplex format, shows
that a multiplex format for the four primers may be possible to detect the three agents in CSF samples. Of 769 CSF samples tested for HRMA and Gram stain / culture methods, 113 (14.7%) were tested positive by HRMA and 29 (3.8%) by Gram stain and / or culture techniques. This shows that HRMA is a much more superior technique for the identification of SP, HI and NM in CSF samples.

Based on the validation with HRMA for APM, same technique was standardized for the molecular detection of M.tbc causing TBM. The primer used was IS6110 which is being used widely in real-time PCT testing. The control strain of M.tbc, H37RV, was used for standardized using the primer IS6110. Dry and wet lab studies confirmed the presence of non-specific cross reactions of IS6110 with a variety of unrelated microbial species and related mycobacterial species. Changing the annealing temperature to temperatures ranging from 60°C to 65°C removed some of the cross reactions, but not completely.

Performing the amplification with a gradient PCR further decreased the cross reactions along with the help of touch-down format; but non-specific amplifications were observed with Staphylococcus lugdunensis and Mycobacterium intracellulare. To get rid of these cross reactions, touch-down format was carried out with pure culture S.lugdunensis; this removed the cross-reaction with the same. Since an isolate of M.intracellulare was not available, cross reaction with this related species could not be substantiated or removed. These results show that IS6110 primer can give non-specific cross reactions in PCR reactions. This together with the reported absence of this genetic element in many Indian M.tbc strains raises the need for another more specific primer for amplification reactions. The Tm value of H37RV was found to be 84.5°C.
Studies on analytical sensitivity of the primer showed that the primer could detect 15 copies/μL of DNA at 10^{-6} dilution indicating its high sensitivity. Robustness study did not show any non-specific interference in the HRMA test system. Inter and inter assay analysis showed some variations in % CV ranging from 0.04% to 6.93%. However these were far below the accepted normal range of values. Of the 109 CSF samples tested for HRMA, 28 (25.69%) was positive for *M. tbc*. Compared with the reported positivity’s for conventional Ziehl-Neelsen stain and culture methods, this high rate of positivity is very impressive. The Tm values of all 28 positive CSF samples were listed to calculate the mean value and standard deviation (SD). The mean value was 84.5°C with a SD of 0.43. Based on the maximum and minimum Tm values observed for the 28 samples 84.5°C ± 3SD was selected for interpretation of a HRMA positive result in clinical samples.

Comparison with 82 samples tested for Gene Xpert, 2 (2.4%) was positive for HRMA while Gene Xpert was positive in 5 (6.1%) samples. This shows higher sensitivity for Gene Xpert. Comparison of the 28 samples positive for HRMA with a commercial kit gave lesser positivity (92.8%). Thus HRMA will be a good adjunct for the detection of *M. tbc* in CSF samples. Since commercial kits are more expensive and often not easily accessible, an in-house standardized HRMA would be very useful and cost-effective adjunct in routine laboratories.

Five most common viruses causing meningitis / meningoencephalitis was selected for the study. These included *Enterovirus* (EV), *Herpes simplex* viruses 1 and 2 (*HSV-1 and HSV-2*), *Varicella zoster* virus (VZV) and *Cytomegalovirus* (CMV). RNA (EV) and DNA (other four agents) were extracted from control cell lines, positive samples or oral polio vaccine
(EV). Appropriate primers reported in the literature was selected and validated using all parameters as summarized for the above two studies. They were all found specific for the intended targets and did not show any cross reactions.

Analytical sensitivity done on the primers and analyzed by LoD determination, probit analysis and linearity analysis showed that they were in the accepted ranges and confirmed their high sensitivity. The Tm values varied from 76.5ºC to 86.5ºC and the melt curves could be easily differentiated from each other. Of the 950 CSF samples tested, 72 (9.6%) were positive for HRMA for various viral agents. Of these, HSV-1 (n = 29) was the highest followed by CMV (n = 15), EV (n= 12), VZV (n = 9) and HSV-2 (n = 3). One CSF sample identified EV + CMV while another one identified HSV-1 + CMV. All 72 samples positive for HRMA were also tested by a commercial kit. HRMA missed two samples that were positive for EV by the kit; both had low viral load. Possibly, the kit had a better sensitivity and was therefore able to pick up even low concentrations of NA.

HRMA is a simple, rapid and robust molecular based test for the recognition of both bacterial and viral agents triggering meningitis. In-house standardization and validation of the primers and test system need to be carried out prior to applying it for clinical studies. Since it does not make use of a fluorescent probe, HRMA is a relatively cost-effective amplification technique. Most modern real-time PCR machines have HRM capability and software and therefore can easily be adapted into laboratories with PCR capability. HRMA can be adapted for the detection of all microbial agents and will be a very useful adjunct in the near future.