CHAPTER 1

INTRODUCTION

1.1. MENINGITIS AND MENINGOENCEPHALITIS

Acute meningitis and meningoencephalitis continued to be a major problem of concern in many countries of the world (Brouwer et al. 2010). It may be infectious or non-infectious; the infectious types can be caused by a variety of bacterial agents including Mycobacterium tuberculosis (M.tbc), viral, fungal and protozoal agents (Bartt 2012; Thakur and Wilson 2018 and Parpia et al. 2016). Rapid diagnosis and early initiation of antibiotic treatment and adjunct therapy were critical to successful outcome (Heckenberg et al. 2014). Most often, the clinical symptoms are overlapping and non-specific so that clinical diagnosis alone cannot give a differential diagnosis (Seth et al. 2017). This will directly influence the treatment protocol which is very necessary to decrease mortality. Inappropriate or delay in treatment resulted an irreversible neurological sequelae (Luoma and Reddy 2012). Although algorithm based diagnosis can help in clinical diagnosis to a large extent, laboratory methods will go a long way in establishing the precise etiological agent. This in turn will help in instituting appropriate treatment protocol at earliest possible time which will save the patient or help to prevent neurological sequelae. Since time is an important factor in all these events, a rapid diagnosis will go a long way in establishing a precise diagnosis at the earliest possible time.
Meningitis is a syndrome of fever, headache and meningismus with inflammation in the arachnoid space and CSF pleiocytosis (Misra et al. 2011). It may be acute, sub acute or chronic according to the time to presentation. Acute meningitis presents itself within hours to days where is chronic meningitis typically takes 4 weeks or longer to present (Baldwin and Zunt 2014). Acute meningitis is most often infectious in nature with a bacterial or viral etiology although non-infectious aetiologies exist for differential diagnosis. Acute bacterial meningitis is a main problem in the developing world where the average frequency is 50 cases per 100,000 populations (Brouwer et al. 2010). Approximately one in 250 children develop bacterial meningitis during the first 5 years of life, with mortality rate greater than 50% younger children. In children <15 years of age, it accounts for 74% of cases, 45% of cases in children < 2 years of age. The triad of agents namely, \textit{S.pneumoniae} (SP), \textit{H. influenzae} (HI) and \textit{N. meningitidis} (NM) account for one third of cases of meningitis and 70% of death in children and adults (Madhumita and Gupta 2011). Members of family enterobacteriaceae are predominantly seen children <24 months of age. \textit{Listeria monocytogenes} and group B \textit{Streptococci} have been reported predominantly from western countries (Chauhan et al. 2015 and Furyk et al.2011). The likely etiological agent of acute bacterial meningitis depends on the age risk factors and underlying disease status of the patient. In resource high countries where vaccination to the above three agents have been successfully employed, viral agents have replaced them as the predominant agents of meningitis.

Tuberculous meningitis (TBM) is the most fatal complication of tuberculosis and forms the predominant form of extra pulmonary tuberculosis (Marx and Chan 2011 and Rock et al. 2008). It arises utmost frequently in children and weakened and immune incompetent adults (Reeves and Swenson 2008). It usually arises from a hematogenous spread of a primary focus of
infection with *M.tbc*. Clinical examination may not differentiate it from acute bacterial meningitis and clinical symptoms may be non-specific; this will warrant a differential diagnosis. A CSF pleocytosis with lymphocytosis has been used to initiate an empirical antituberculous treatment. CSF protein concentration is normally high and the sugar concentration is normal. However these values show variations and cannot be used for a specific diagnosis of TBM.

Detection of *M. tuberculosis* complex (MTBC) generally relies on a combination of *in vitro* cultivation and staining of acid-fast bacilli on unprocessed sputum smears with conventional light microscopy. However, the sensitivity has been variable (range of 20–80%). Even in well-equipped clinical laboratories, approximately 3 weeks are required for the detection of TB cases using liquid culturing systems. With the advent of molecular biological techniques, there have been significant advances in DNA amplification and hybridization that are helping to rectify existing flaws in the diagnosis of TB. The detection of mycobacterial DNA in clinical samples by PCR is a promising approach for the rapid diagnosis of TB (Tortoli et al. 2012). This molecular diagnostic method is not only easy to apply, but also allows for direct detection of bacteria in the clinical specimen. Their major limitation is poor sensitivity with paucibacillary specimens such as microscopy-negative and extra pulmonary samples (Parpia et al. 2016). Several generations of tests, based on different technologies, have been introduced in the past 15 years (Harries and Kumar 2018).

With increased availability and usage of vaccines in high income countries, the incidence of acute bacterial meningitis has drastically come down (Amare et al. 2018). Consequently, there had been a corresponding increase in
the incidence of meningitis to certain viruses. Although the exact magnitude of the problem is not clearly understood, it is known that herpes and enteroviruses are the most common agents of viral meningitis. Epidemics due to Enteroviruses and Japanese B encephalitis viruses have been known in South East Asian countries for many decades. Mortality due to viral encephalitis is much higher as agents for antiviral treatment are extremely limited in number (Tunkel et al. 2008).

Meningitis and meningoencephalitis due to fungal and protozoal agents are relatively fewer and they occur in patients with lowered immune status (Sharma 2010). A differential diagnosis from other forms of meningitis and meningoencephalitis based on clinical symptoms is often difficult and therefore laboratory methods need to be employed to pinpoint the precise etiology. As stated earlier, clinical symptoms alone cannot make a diagnosis of meningitis between the different etiologies and therefore much emphasis is now put on laboratory methods of diagnosis.

Conventional methods for laboratory diagnosis of meningitis include cytology, protein and sugar estimations, microscopy and bacterial culture methods. Although lymphocytosis can differentiate a case of TBM from ABM, cytological features of ABM may not be characteristic enough to give a definite diagnosis. Protein and sugar concentrations show considerable variations and therefore are not diagnostic enough to give a differential diagnosis. Therefore it is very important microbiological methods that help to identify the precise etiological agent/s are necessary to give a correct clinical diagnosis.

One of the most widely used and validated tool in diagnostic microbiology is the conventional microscopical techniques (Lehman 2003).
Easy to perform and able to get a result in a very short span of time, Gram stain is globally accepted as a standard technique. Although a negative Gram stain does not rule out a clinical disease, a positive result will go a long way in helping to initiate appropriate treatment to the patient. Presence of bacteria in the presence of number of pus cells is the hallmark of acute pyogenic meningitis (APM). Therefore Gram stain remains as the first step in the microbiology laboratory. Although widely used, conventional cultural methods are time consuming and therefore do not help in a differential diagnosis; without this, proper treatment protocols cannot be put in place. For these reasons, rapid antigen detection methods including immunofluorescence, latex and co-agglutination methods were used for many years. However lack of standardization due to batch-to-batch variation, these methods did not find wide support over time.

With the advent of nucleic acid based amplification tests (NAATs), especially polymerase chain reaction (PCR), molecular based tests have carved a niche in the diagnostic armamentarium (Caliendo et al. 2013; Cobo 2012; Maurer et al. 2017 and Buchan and Ledeboer 2014). Qualitative PCR which is relatively easy to perform and more-cost effective is based on the detection of amplicon by nucleic acid staining under UV light. Over time, this was replaced by quantitative or real-time PCR (Q-PCR) which is based on the real-time enumeration of nucleic acid (NA) molecules at the end of every cycle of amplification. This has the advantage of determining the actual number of microbial NA molecules by using fluorescent probes which can work out to be expensive. Several other modifications of PCRs are available, but due to the ease of performance and interpretation, Q-PCR has found very wide acceptance.
High resolution melt analysis (HRMA) is an adaptation of real-time PCR and was introduced in 1997 (Thomsen et al. 2012 and Wittwer et al. 2003). It is a natural extension of continuously monitored PCR within each cycle. It is a simple and fast method based on the melt temperature (Tm) of DNA molecule during its replication. Tm is the temperature at which 50% of the DNA is polymerized and is very specific to each microbe, often to the species level. Melting curve techniques is facilitated by the current availability of improved double-stranded DNA binding dyes along with next-generation real-time PCR instrumentation and analysis software. HRMA can discriminate DNA sequences based on their composition, length, GC content, or strand complementarity. Unlike most DNA analysis techniques, no processing or separations are necessary. The stability of DNA duplexes is monitored by the fluorescence of SYBRs Green I as the temperature of the solution is increased. In due course, HRMA has been used as a rapid method for genotyping, known variants or scanning for unknown variants.

Like other Q-PCR techniques, HRMA has high sensitivity, specificity, rapid turn-around time and does not require a specific fluorescent probe for final detection (Ruskova and Raclavsky 2011). This cuts down the cost considerably and therefore makes it an ideal test for the diagnosis of meningitis / meningoencephalitis in resource crunch situations. HRMA has been used successfully for the diagnosis of a variety of agents causing infectious diseases including bio threat and other clinically relevant bacterial species. Against this background, a study was undertaken to standardize an in-house HRMA technique for the molecular detection of important bacteria and viruses causing meningitis / meningoencephalitis in India.
1.2. **OBJECTIVES**

Overall objective in this study is to detect pathogen DNA in cerebrospinal fluid using HRMA.

**Specific aims are**

To Standardize and validate HRMA for the detection of *Streptococcus pneumoniae (SP)*, *Haemophilus influenzae (HI)* and *Neisseria meningitidis (NM)* from cerebrospinal fluid using appropriate specific primers

To Standardize and validate HRMA for the detection of *Mycobacterium tuberculosis* from CSF of patients with meningitis / meningoencephalitis using the primer, *IS6110*

To Standardize and validate HRMA for the detection of *Enterovirus (EV)*, *Herpes Simplex virus 1 / 2 (HSV-1 & 2)*, *Varicella Zoster virus (VZV)* and *Cytomegalovirus (CMV)* from CSF of patients with suspected meningitis / meningoencephalitis.

1.3. **THE IMPORTANCE OF THE PROJECT AREA**

Standardization of HRMA for detection of nine different microbial pathogens causing meningitis / meningoencephalitis showed specific melt curves and Tm values. Thus the specific etiology of meningitis / meningoencephalitis can be easily differentiated by HRMA. The system works in a Real time - PCR platform and therefore can be adapted by any laboratory which has a PCR capability.
The real-time PCR and melting curve analysis optimized have high sensitivity and short turnaround time in clinical samples. The highly specific and less labor intensive molecular assay in clinical laboratory may help the speedy diagnosis and prompt treatment of patients with severe critical illnesses. Thus HRMA can help in the diagnosis even when culture is negative due to prior antibiotic treatment which is often the case in India. Multiplexing the PCR technique could make HRMA even more useful in a clinical setting.

Since HRMA does away with probes for the detection of amplicon, it works out much cheaper than the real-time PCR. Like other PCR protocol, HRMA has also has a very short turn-around time, high sensitivity and runs on an open PCR platform. As this study has shown, HRMA can be standardized to identify totally unrelated microbial DNA or RNA. All tests were carried out in a uniplex format except for NM, where a biplex format was used. This shows that with proper standardization, even a multiplex format may be employed so that multiple microbes can be detected from the clinical sample at any given point in time (Leber et al. 2016 and Hanson et al. 2016).

Technology adoption will not occur without robust clinical intervention and cost effectiveness studies. Initial clinical validation showed low cost, short turnaround time, speed and high accuracy indices (Sensitivity, Specificity, PPV and NPV). It is a very good adjunct to conventional culture techniques. With adequate standardization and testing of samples with more stringent criteria, HRMA can, in future, even replace the conventional techniques for the diagnosis of APM. Same technique has been standardized for the diagnosis of meningitis of other microbial etiology, septic arthritis, rheumatoid arthritis, glomerulonephritis, Pyrexia of Unknown Origin (PUO),

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tropical fevers, Sexually Transmitted Diseases (STD), Blood Born Viruses (HBV, HCV & HIV) and Respiratory infections.