Chapter 5

Discussion

Respiratory tract infections caused by hMPV and HBoV are a major cause of concern in the health of infants and young children (Schildgen et al. 2008). Studies have been reported in different countries on the involvement of both hMPV and HBoV in respiratory tract infections of children (Smuts et al. 2008; Milder and Arnold, 2009; Pilger et al. 2011; Zappa et al. 2011) and in immunocompromised adults (Muller et al. 2009).

This study presents data on the frequency of hMPV and HBoV infections in a prospective cross-sectional study among children ≤5 years of age.

Nasopharyngeal swabs were collected in majority of the studies related to respiratory viruses, especially hMPV and HBoV. Though, Lambert et al. (2008) found a slightly higher rate of sensitivity for nasopharyngeal swab (95%) compared to throat swabs (90%) in the isolation of human metapneumovirus, in this study, the collection of the nasopharyngeal sample was difficult in terms of both non-compliance by the parent and non-cooperation by the child. The collected samples in our study were also not satisfactory. In a literature survey done by us, we found that this approach would be satisfactory for detection of respiratory viruses (Lieberman et al. 2009, 2010). A recent study by Kim et al. (2011) showed oropharyngeal swabs to be more sensitive than nasopharyngeal swabs for the detection of respiratory viruses. In this cross sectional study, oropharyngeal samples were collected for the detection of these two viruses. The age and the area of inhabitance of the patient were recorded to assess the distribution of these viruses in the children of different age groups under 5 and domiciliary status.

König et al. (2004) found that coinfections with RSV and hMPV are more severe than infections with either RSV or hMPV alone, at least in children younger than 3 years of age. The epidemiological and clinical features of hMPV infection were similar to those of RSV infection in terms of clinical severity and pattern (Wilkesmann et al. 2006). In infants and children with respiratory tract infection, viral diagnosis from respiratory secretions becomes inevitable not only because any clinical, laboratory,
and radiological signs cannot adequately differentiate viral from bacterial infections. Also, these infections may lead to complications like pneumonia and can also trigger asthma and cause serious health problems. Clinical signs like respiratory rate with history of rapid breathing and presence of chest retraction were found to be specific and sensitive indicators of LRI in children. This could be used at the community level for prophylactic antibiotic therapy to prevent secondary bacterial pneumonias which significantly reduces the mortality rate in children (Cherian et al. 1988). It is important to know the etiology of presumptive viral infections to devise future strategies. Our study specially focused on these two viruses.

Cote et al. (2003) evaluated detection of hMPV using real-time PCR assays targeting different regions (the N, M, L, F, and P genes) but the N and L genes that code for internal viral proteins were found to be particularly suitable for hMPV detection with high specificity and sensitivity. In this study the N gene of hMPV was amplified using semi nested RT-PCR assay from respiratory samples.

The viral capsid proteins (VP1/VP2) and non-structural proteins (NS-1 and NP-1) coding genes are generally used as the target gene for the detection of HBoV. Schildgen et al. (2008) however found the VP1/VP2 to be a highly variable region and NS1 and the NP-1 genes to be more conserved. N gene for hMPV and NP gene for HBoV were therefore used for both amplification and sequence confirmation in this study.

Though direct fluorescent antibody test and rapid cell culture can be performed for hMPV, they are not routinely done (Ginocchio and McAdam, 2011). Molecular assays such as PCR have been widely used for the detection of these viruses in respiratory samples. Respiratory virus detection is highly dependent on the type of sample collected apart from time of collection, age of patient and storage of samples prior testing. Samples like nasopharyngeal aspirates, nasopharyngeal washes, and nasopharyngeal swabs are reported to be best samples (Ginocchio and McAdam, 2011).

Maggi et al. (2003) demonstrated hMPV in nasal swabs and in blood-plasma by PCR with a highest positive rate of 43%. Nasopharyngeal swab have a slightly higher rate.
of sensitivity compared to throat swabs (90%) in the isolation of hMPV (Lambert et al. 2008). The collection of the nasopharyngeal sample was difficult in terms of both non-compliance by the parent and non-cooperation by the child. Thus instead of mere throat swabs, oropharyngeal swabs were collected. This in our opinion could be of adequate sensitivity for the virus detection. This approach has been found to be satisfactory for the detection of respiratory viruses (Lieberman et al. 2009, 2010). Kim et al. (2011) recently assessed the relative measures of performance for nasopharyngeal and/or oropharyngeal swab specimens from pediatric and adult patients. The two methods did not differ significantly for hMPV, and other viruses like influenza A (H3N2) virus, PIV-1, or RSV.

Guido et al. (2011) identified hMPV and HBoV with a positive rate of 18.9% and 45.7% respectively in oropharyngeal swabs collected from children. The authors used nested PCR for both hMPV and HBoV.

In this study, the frequency of hMPV and HBoV was 12.7% and 0.67% respectively. The frequency of hMPV in our study is consistent with other studies reported from other parts of the world. A semi-nested PCR approach was used for the detection of hMPV whereas non-nested format was used for HBoV. The low positivity rate of HBoV could be due to the non nested type of PCR study undertaken. However, only one more positive was picked up by the nested PCR in the fifty subset samples. Hence it is believed that HBoV may be low in this study population.

In this study, the number of hMPV positives was higher from samples collected from VGMCH compared to other sites. The number of samples collected from VGMCH is higher than other sites; we believe that such government hospitals are generally preferred by the patients at acute stage of infection particularly from rural community as majority of the patients are from the low socioeconomic status groups. The difference in frequency of hMPV positives between the two centers (VGMCH and SNH&RC) however was statistically insignificant ($\chi^2=1.76; p=0.18$).

The risk factors for viral respiratory infection are prematurity, bottle feeding, overcrowding, domestic smoke pollution due to the use of woodstove, and lack of sinks in the household (Bulkow, 2012; Chauhan and Johnston, 2003; Pandey et al.
1989) which are very commonly seen in Indian rural areas. In this study, patients from rural area were higher compared to peri-urban area.

Despite the fact that viruses cause most of the respiratory illnesses, the needless use of antimicrobial agents in hospitals as well as over the counter is commonly seen in community practice in rural areas for the treatment of acute URI and LRI. Widespread antimicrobial use in the treatment of hospitalized patients creates an ideal environment for the development of antimicrobial resistance (Shiley et al. 2010). This could be limited by the use of PCR assays that establish the cause of ARI in children including hMPV and HBoV. Rapid identification of viral infections can furthermore help control secondary bacterial community-acquired pneumonia and nosocomial transmission (Berger et al. 2010).

hMPV was reported to be one of the important etiologic agent of LRI i.e. in bronchiolitis, exacerbations of asthma/wheezing, pneumonitis in transplant recipients and rarely pneumonia (Chen et al. 2010; Pavia 2011; Anderson et al. 2012). Manoha et al. (2007) found no difference in the prevalence of bronchiolitis in children with hMPV, RSV and rhinovirus. Though hMPV was also reported to be associated with URI (Williams et al. 2006; Garcia-Garcia et al. 2006), such reports either presented a very low prevalence rate (i.e. 5%) or the virus was more commonly associated with LRI than URI. The hMPV infection with URI was reported to be restricted in hMPV reinfections in adults (Boivin et al. 2007; Pancer et al. 2011). HBoV is largely reported to be one of the important causative agents primarily of LRI in children (Ma et al. 2006; Zhang et al. 2008; Ghietto et al. 2012). In this study, we included both URI and LRI as inclusion criteria, and compared the positive rate of in different age groups; the difference however was statistically insignificant in any age group. The HBoV detection was low yet for comparison.

The hMPV positive rate was reported to be higher in early summer (Williams et al. 2006; Talavera and Mézquita 2007; Aberle et al. 2010) and few others have shown the virus in winter months (McAdam et al. 2004; Regev et al. 2006). In this study, we found higher positivity rate of hMPV during both cooler and wet months of the season. The varying pattern of hMPV circulation may be due to different climatic and demographic differences.
Apart from pneumonia and bronchiolitis, symptoms like wheezing, asthma, difficulty breathing, and sore throat were reported to be more common in hMPV infections (Williams et al. 2010; Schildgen et al. 2011). In this study, we found dry cough and cough with secretions, coryza and rhinitis to be the most common clinical features in hMPV positives compared to hMPV negatives. However, this difference was statistically not significant for any symptom. Each symptom however was compared individually between hMPV positives and negatives in each different age group. Coryza was significant in children above 2 years and rhinitis in children over 1 year of age. Vomiting and productive cough was significant in the age group of 2 to 3 years and more than 3 years respectively.

Hajjar et al. (2011) found the incidence rate of hMPV to be 8.3% among Saudi children. The infection was prominent during the autumn and winter. Zhu et al. (2011) found a prevalence of 4% hMPV infection in children in Beijing in a surveillance study during a four-consecutive year period. The peak of hMPV activity mostly occurred in late spring and majority of them had LRI. Most of hMPV positive children were under five years old. The respiratory viral pathogens detected most frequently in children were rhinovirus, followed by respiratory syncytial virus, HBoV, and human metapneumovirus by real-time PCR or direct immunofluorescence testing (Ursic et al. 2012). Caracciolo et al. (2008) found a high incidence of hMPV infection (25.3%) in nasal washes obtained from children younger than 5 years of age during the 2005-2006 winter-spring seasons in Italy.

Bharaj et al. (2009) found hMPV in 3.6% of children by a multiplex PCR for viruses in North India. In a recent study from the same hospital by Banerjee et al. (2011), the seroprevalence of hMPV was higher in adults compared to children less than 5 years of age. However, the sample size of the latter study was too small in each age group. Moreover, the antibody detection could always be less sensitive than RNA detection in respiratory. Banerjee et al. (2007) reported 12% of hMPV infections in North India and Agrawal et al. (2011) reported 5% in eastern India.

To date, there is only limited data on hMPV among children available from India and this article is the first to report on the frequency of hMPV and HBoV from India (South) particularly among rural and peri-urban children.
In this study, the youngest age at which hMPV positive recorded was a 5 months old child. Four patients had wheezing but none of the positive patients had asthma, rashes or diarrhea. Rawlinson et al. (2003) found hMPV less frequently in children with asthma. Fujitsuka et al. (2011) also found hMPV to occur very rarely in Japanese children with acute wheezing illness. The link to the induction of wheezing and exacerbation of asthma is still not clear.

In this study, the detection limit of the semi-nested PCR targeting the N gene was $6.69 \times 10^5$ plasmid copies per reaction. Ali et al. (2011) reported the Respiratory MultiCode-PLx multiplex assay to be more sensitive than individual real-time RT-PCR assay for the detection of 11 common respiratory viruses including hMPV. The assay though very rapid and sensitive, is highly expensive and not commonly available in developing countries like India.

In the phylogenetic analysis, the genetic diversity of 38 hMPV positive samples obtained from this study was examined.

hMPV N and P together are required for the formation of cytoplasmic inclusion bodies, the N terminus of hMPV N is not essential for binding to hMPV P but is required for the formation of viral inclusion-like complexes. This indicates the importance of N gene in viral pathogenicity and hence becomes important while looking at genetic diversity at nucleotide and amino acid level of this gene.

Different genes of hMPV have been used as the targets by the researchers for PCR amplification and phylogenetic analysis. We have used N gene for both amplification and phylogenetic analysis. Genetic diversity was found to be low amongst the conserved F gene sequences but very high amongst G gene sequences. Agarwal et al. analyzed respiratory samples for hMPV by RT-PCR assay targeting N gene. On the phylogenetic analysis of G and F gene sequences, the latter was found to be more conserved than the former. Agapov et al. analyzed the amino acid sequences of different genes of hMPV. They found maximum differences in the G gene (60%–63% identity within a genotype from year to year), significant but fewer differences in the SH gene (87%–89% identity within a genotype), and high conservation of the F and N genes. This indicated that N gene would be an ideal target for PCR and nucleotide
sequencing as well especially in identifying geographically circumscribed circulating strains of the virus (topotype).

Phylogenetic analysis based on the 38 partial nucleotide sequences of the N gene of hMPV positive samples revealed the presence of three genetic lineages. The positive samples obtained in each sampling site and the seasonality of the hMPV was consistent in each cluster suggesting the circulation of closely related strain(s) (topotype) in the respective communities.

In this study, all available hMPV N gene sequences available in GenBank database in each subtype and sub lineage from different countries were retrieved and a consensus sequence was established for each country. These consensus sequences of the subtypes were used for comparing our strains. The phylogenetic analysis showed clear division of subtypes closely matching with respective global strains.

In a comparative distribution of hMPV subtypes globally from 2001 to 2009 by Li et al. (2012) A2 particularly A2b subtype is shown to be highly prevalent and B1, B2 and A1 being very less prevalent. On the contrary, we observed higher number of B2 strains (71.1%) followed by A2b (18.4%), A2a (7.9%) and B1 (2.6%) strains. About 90% of the strains belonged to B2 and A2b which is similar to the studies reported in Japan and South Africa. In this study, we found all subtypes except A1 indicating the co-circulation of multiple subtypes and sub-lineages within the population.

hMPV infection occurs in adults of all ages and may account for a significant portion of persons hospitalized with respiratory infections during some years. Walsh et al. (2008) reported the proportion of asymptomatic infections in large adult cohorts to be 44% among the healthy elderly group, and 39% in the high-risk group. Though the period of virus shedding has not been studied in adults, it is reported to be up to 3 weeks in nasal secretions in children. von Linstow et al. reported the excretion of hMPV RNA in different secretions like nasal secretions, saliva, and sweat, as possible modes of transmission. In contrast, asymptomatic shedding of hMPV in children is very less (1.2%). However, a complete understanding of how the virus transmits between humans is lacking, and whether human-to-human spread alone accounts for the seasonal emergence of epidemics has been questioned. Moreover, since travel and
commerce expanded in recent years, more and more people travel internationally particularly from Canada, China and Singapore, hence more cases of asymptomatic hMPV shedders are likely responsible for the spread of multiple genotypes of hMPV infection into a population. Children are likely to contract the infection from the infected adults (asymptomatic shedders). This could explain us encountering four of five known subtypes of hMPV in our population.

Respiratory viruses have been shown to be as a result of imported infections. Air travel can certainly influence the global spread of emerging and established infectious disease. hMPV infections may be spread on the aircraft as in the case of spread of severe acute respiratory syndrome (SARS) and avian pandemic of H5N1 influenza. Since international commerce and tourism related travel is an increasing phenomenon, new strategies to prevent the spread of emerging respiratory viruses like hMPV should be considered.

The high degree of sequence identity and the resulting close clustering of majority of our study strains potentially indicate the relative homogeneity of the N gene sequences in the virus population. The sampling sites in this study were all within 15 km range and the sampling period was over a year (Nov 2010 to Nov 2011). However, further surveillance over an extended period with examination of more isolates from these areas is necessary and the divergence at different genomic regions have to be analyzed to better understand the nature and extent of the sequence divergence of hMPV within the populations in India (South).

In this study, the strains were homogenously clustered with global strains of respective subtypes. All the subtypes were closely related to the strains from Canada, The Netherlands and Australasia region (India, Australia, China, Japan, Taiwan and Singapore). Such close clustering of hMPV isolates suggests the temporal rather than geographic variation in the evolutionary pattern of hMPV strains.

Phylogenetically, RSV is the closest human virus related to hMPV, seasonal epidemiology and the clinical symptoms of hMPV may share an overlapping spectrum with RSV. The genetic diversity in the hMPV genes are reported to lead to antigenic variations and the hMPV genetic clusters may also represent different
antigenic groups. The changes in amino acid sequences may benefit the virus, possibly by modifying epitopes and allowing it to escape pre-existing immunity. Phylogenetic analysis can provide information on how the viruses are disseminated or the pattern of spread in a region or even its source.

Generally, in RNA viruses, the small compact genomes and high mutation rates and the altering environments in which they replicate create the conditions for robustness, which is advantageous to the virus. hMPV does not appear to exhibit progressive genetic evolution, unlike influenza virus that exhibits rapid genetic drift associated with antigenic variation resulting in immune escape. RNA viruses mutate frequently due to the infidelity and lack of proofreading ability of RNA-dependent RNA polymerases. The hMPV polymerase has been shown to produce frequent errors resulting in the circulation of field strains with nucleotide variations at a similarly high rate. The mean rate of nucleotide substitution for the N gene was found to be $8.5 \times 10^{-4}$ (2.0–15.0$ \times 10^{-4}$) substitutions per site, per year.

To detect signatures of positive and negative selection in partial N gene sequences of hMPV strains obtained in this study, IFEL approach was used. Though REL was reported to be the most powerful than FEL and IFEL because it uses the entire alignment to make inferences about rates at each site, it generally has the highest rate of false positives. IFEL test is also shown to be a conservative test, and produces the lowest false positive rates of all. The IFEL analysis is reported to be the more specialized program to test sequence alignment from multiple viruses obtained from a population.

Lo Presti et al. (2011) analyzed 435 bp fragments of the F gene of hMPV. In F glycoprotein dataset, they observed only two positively selected sites with a $\omega$ (Omega or dN/dS ratio rate) value of 1.408 and 1.429, respectively, and 27 negatively selected sites. But the N gene of hMPV has not been analyzed adequately.

Positive selection analysis on nucleoprotein gene sequences has been performed previously for many other viruses. In hMPV, when the mean values of $\omega$ (dN/dS) of the G, F and N genes were compared between the different hMPV lineages, it revealed limited positive selection and abundant negative selection for the G (mean
Neither positive nor negative selection was detected for the N protein (mean $\omega=0.038$) using the REL analysis. Lo Presti et al. (2011) also found abundant number of negatively selected sites at the partial F gene sequences and less number of positively selected sites in both REL and FEL approaches. Similarly, in this study on partial N gene sequences, in REL and FEL analysis, there were no positively selected sites and in IFEL, there was only one positively selected site. But we observed a significant number of negatively selected sites in all REL, FEL and IFEL approaches. This is consistent with our phylogeny results which showed hMPV strains were homogenously clustered away from the global strains suggesting occurrence of natural selection upon the N gene of hMPV strains. The only positively selected site was not considered as an evolutionary “hot spot” because it was not consistent with REL and FEL analysis. However, our data on the occurrence of abundant negatively selected sites reflect a high degree of conservation of N protein. Highly conserved proteins are generally often required for basic cellular function, stability or reproduction. Our data suggests that the highly conserved nucleotide sequences of N gene have important functional value probably necessary for viral infection through the formation of cytoplasmic inclusion-like complexes as described by Derdowski et al. (2008).

Suzuki (2004) showed all surface proteins of poliovirus to be negatively selected and no positively selected sites. The authors also suggest that vaccines directed against epitopes, which consist of negatively selected sites protect vaccines more effectively than those directed against epitopes which contain positively selected sites. Thus, through molecular evolutionary analysis of a particular viral protein (and its coding nucleotide) sequences, the candidate epitopes for immunization could be predicted which consist of negatively selected amino acid sites.

RNA viruses are characterized by abundant genetic variation and short replication time. They utilize all possible mechanisms of genetic variation to sustain their survival. The transient deleterious mutations in RNA viruses are later eliminated by negative selection otherwise called as purifying selection. Because more genomic changes are harmful than are beneficial, negative selection plays an important role in maintaining the long-term stability of biological structures by removing such deleterious mutations and in the evolution of a viral quasispecies.
It is important to note that negative selection plays a vital role in molecular diversity. Climate change and other habitat alterations of South India could have currently placed these strains under such negative selection. Further understanding the causes, extent, and consequences of negative selection can contribute important insights toward the molecular diversity of hMPV in South India.

However this study had a few limitations. The patients shown to be positive for hMPV or HBoV were not tested for other viruses such as respiratory syncytial virus for co-infections.

We have demonstrated that hMPV is associated with a considerable number of ARI cases. Whereas, relatively lower number of HBoV infections were observed in young children in South India. Our data underlines the role of hMPV in the causation of ARI in south Indian children ≤5 years of age from rural and peri-urban population. Further studies are needed for the better understanding of the epidemiology of these viruses in our population in large field and hospital based studies which screens for a number of respiratory viruses.