Chapter 1

Introduction

1.1 ACUTE RESPIRATORY TRACT INFECTION

1.1.1 DEFINITION
Acute respiratory infections (ARIs) include infections in any area of respiratory tract and associated structures (paranasal sinuses, middle ear and the pleural cavity) lasting less than 30 days (WHO 1990). Respiratory infections can be bacterial or viral in origin, the latter being more common, and are mostly limited to the upper respiratory tract. ARIs can be classified as upper respiratory tract infections (URIs) or lower respiratory tract infections (LRIs) based on the organs affected. Based on the clinical severity ARI can be classified into mild, moderate and severe types (Stefaan 1997).

URI can be defined as an acute febrile illness with cough, coryza, sore throat, or hoarseness, which are very common in the community and are one of the major reasons for hospitalization, particularly during the winter and wet season. The URI include rhinitis or coryza, pharyngitis and laryngitis. The frequency of URI can be six to eight episodes per year and even more in children attending day care centres and school. Majority of these URIs are mild in nature, self limiting and not often life threatening. The URI in infants can cause lethargy and poor feeding. It can also lead to clinical conditions like acute otitis media, asthma exacerbations, and LRI such as bronchitis, bronchiolitis and pneumonia.

LRI is defined as an acute illness (present for 21 days or less), usually with cough as the main symptom, with at least one other lower respiratory tract symptom (sputum production, dyspnoea, wheeze or chest discomfort/pain) with no other complications (e.g. sinusitis or asthma). The clinical symptoms of LRI are tachypnea, fever, cough, hypoxia, bronchitis, bronchiolitis and pneumonia. The changes in chest radiographs observed are infiltrates, hyperinflation, and peribronchial cuffing. Majority of the severe illness and death in ARI is due to LRI especially pneumonia (Rudan et al., 2008). The severity of the disease is more in children less than 5 years of age, the aged and the immunocompromised.
1.1.2 PREVALENCE

Acute respiratory tract infections (ARI) are a major cause illness worldwide and result in hospitalization of infants and young children in developed countries and death in developing countries (Murray et al., 2001). ARI is more prevalent among children, elderly and the immunocompromised patients. There are 10.4 million deaths in children under five years of age worldwide. Among this 4.7 million (45%) occur in the African region, and an additional 3.1 million (30%) occur in the South-East Asia. The death rate per 1000 children aged 0–4 years in Africa and is almost double that of the Eastern Mediterranean region and more than double that of any other region. The two leading communicable disease killers in all regions are diarrhoeal diseases and respiratory infections. ARI account for 4.2 million deaths (7.1%) and are the third leading cause of death globally (WHO 2004). Among the 1.9 million childhood death per year in the developing countries 20% are from India (Williams et al., 2002).

The prevalence of ARI among children in developing and developed countries is the same, but the mortality rate is much higher (30-50%) in the developing countries when compared to that of the developed countries (Broor et al., 2007). The risk factors of severe ARI in developing countries are malnutrition, low birth weight, passive smoking, non-breastfeeding, low socioeconomic status, overcrowding, paucity of health services, lack of awareness, immunodeficiency and HIV infection (Peat et al., 2001; van den Hoogen et al., 2001; Simoes 2003; Prajapati et al., 2012). The prevalence of ARI in developing countries like Kenya, Philippines, Thailand, Colombia, Nigeria, Uruguay etc, was reported in the range of 21.7 to 40%. There is an estimated annual occurrence of 300 million episodes of ARI in India, of which 30 to 60 millions are moderate to severe ARI episode. ARI is responsible for about 30-50 % visits to health facilities and for about 20-40 % admissions to hospital. ARI is the leading cause of mortality and morbidity in India especially in children less than five years of age (Prajapati et al., 2012).

1.1.3 AETIOLOGIES

A wide range of microorganisms including bacteria, viruses, fungi and protozoa can cause respiratory tract infections, the common being bacteria and viruses. Viruses are responsible for the majority of the upper respiratory tract infections, while bacterial infections can be primary or secondary to measles, influenza, or RSV infections.
The common bacteria known to cause acute respiratory tract infections are *Streptococcus pneumoniae*, *Haemophilus influenzae* (type B), *Streptococcus pyogenes* and *Staphylococcus aureus*. Other pathogens are *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* which causes atypical pneumonia (Grondahl et al., 1999).

ARI due to viruses are an important cause of morbidity and mortality (Fauci and Morens 2012). The major viral etiological agents of ARI in all age groups include respiratory syncytial virus (RSV), influenza viruses, parainfluenza viruses (PIV), adenoviruses, human rhino virus (hRV), human metapneumovirus (hMPV), human bocavirus (HBoV), corona viruses and picornaviruses (Grondahl et al., 1999; Weigl et al., 2000; Bezerra et al., 2011). Among these RSV, hMPV, hRV and PIV predominate as the cause of ARI in children less than five years of age and has a seasonal occurrence with or without co-infection (Brunstein et al., 2008; Regamey et al., 2008; Kaplan et al., 2008; Sung et al., 2009).

**1.1.4 DIAGNOSIS OF VIRAL RESPIRATORY TRACT INFECTIONS**

The differentiation of the etiological agent for respiratory tract infection based on the clinical conditions is practically impossible. Rapid and precise diagnosis of the etiological agent is very much essential in the treatment and control of the spread of viral respiratory infections. Laboratory diagnosis can have a significant positive effect in improving the patient care based on the appropriate diagnostic method chosen by the health care personnel in performing the test. There are various methods for the detection of respiratory tract infections namely rapid antigen testing, immunofluorescence tests for antibody detection, conventional and rapid cell culture methods and molecular based nucleic acid amplification assays. The various specimen types for the detection of the respiratory viruses include nasopharyngeal aspirates, nasopharyngeal washes, nasopharyngeal swabs and oropharyngeal swabs in viral transport media, sputum, endotracheal aspirates and bronchoalveolar lavages. The sensitivity of each method depends on factors such as sample type, time of sampling since the onset of the symptoms, patient age, antigen target and the properties of the virus (Ginocchio 2007). Immunocompromised patients shed low titre of virus over extended period of time making it difficult for its detection by non molecular methods. Hence nucleic acid amplification by molecular based methods has become
more popular for the identification of the respiratory viruses as they are rapid and most sensitive assays. The various methods for the identification of the respiratory viruses have its own advantages and drawbacks.

1.1.4.1 VIRUS ISOLATION IN CELL CULTURE
Isolation of the virus in cell culture is still considered as the “gold standard” in viral diagnosis, though it is time consuming and laborious. Moreover the virus isolated in culture is required for further identification. It is also a very sensitive method as it can detect a single infectious virion. The disadvantages of cell culture method are that they are time consuming, requires technical expertise, need different cell lines for even strains of the same line and also need alternative method for the detection of viruses that do not show any cytopathic effect in cell culture.

The three major types of cell culture are primary, diploid and continuous. The primary cell cultures are prepared from animal tissues. The cells are separated into single cells, treated with proteases and suspended in culture media. It is then transferred to suitable flasks and maintained. The cells have a limited lifespan. The most commonly used primary cell cultures are derived from monkey kidneys, human embryonic amnion or kidneys and chicken or mouse embryos. Homogenous population of cells which has the capability of dividing up to 100 types is used in diploid cell cultures. The most commonly used diploid cells are established from human embryos, such as WI-38 strain derived from human embryonic lung. Continuous cell lines are derived from mostly tumour tissues or by the treatment of primary and diploid cell lines with mutagens. The commonly used cell lines are HEp-2 (human epithelial) and HeLa (Henrietta Lacks) cells derived from human tumour tissues; Vero cells from African green monkey kidneys; and BHK-21 (baby hamster kidney) cells from hamster kidneys. A positive result on culture is indicative of the presence of viable virus in the sample. The virus can be isolated and used for drug susceptibility assays and for determining the pathogenicity. Shell vial culture is the method where there is combination of centrifugation and inoculation of the specimen on cell mono layers followed by incubation for a certain period of time. The virus is detected specifically by labelled monoclonal antibodies directed against viral proteins which are produced soon after infection of the cell.
The advantage of shell vial culture over conventional culture is that it is more specific, less time consuming and require less technical expertise in reporting the results. The main disadvantage of this technique is that only specific viruses that are the target can be detected thereby missing out the novel viruses. The effective isolation of the virus by cell culture depends on the proper selection, collection, storage, transport and preservation of the sample (Leland and Ginocchio 2007).

1.1.4.2 ANTIGEN DETECTION
Rapid identification of the virus can be performed by determining the specific protein present in the sample. They are commonly used for the detection of influenza and respiratory syncytial viruses. This can be achieved by applying either a specific fluorescein-labelled antibody in direct fluorescent assay (DFA) or a specific primary antibody and a labelled antispecies antibody in indirect fluorescent assay (IFA) to the sample fixed on a microscope slide. The indirect method may be more sensitive, because more labels is bound to an infected cell. DFA is commonly the preferred method of screening for a variety of respiratory viruses due to the ease in performing the test and the rapid availability of the results. However the test has certain limitations such as poor sensitivity when compared to the nucleic acid amplification and the conventional culture methods and as a result the negative results must be confirmed with the culture and nucleic acid amplification techniques (Rahman et al., 2008; Loeffelholz and Chonmaitree 2010).

The viral antigens can also be detected by Enzyme Linked Immuno Sorbant Assay (ELISA) methods. It enables the detection and quantification of the antigen present in the sample by visualizing the colour reaction. It can also detect the cell free antigen and can be automated providing more accuracy to the test (Semple et al., 2007). Recently simple and rapid assay methods like membrane ELISA and lateral flow immunochromatography assays are available but their sensitivity is poor when compared to other tests like DFA and culture (Grijalva et al., 2007; Hurt et al., 2007; Uyeki et al., 2009). The rapid tests are more sensitive in children than the adults. This may be due to the more amount of virus shed by the children during infection than the adults (Loeffelholz and Chonmaitree 2010). Other serological assay methods such as neutralization test, hemadsorption inhibition test, hemagglutination inhibition test, and
complement fixation test are some of the traditional serological methods for the identification of respiratory viral pathogens.

1.1.4.3 MOLECULAR METHODS
The detection of respiratory viruses by nucleic acid amplification techniques (NAAT) has become popular as the nucleic acid (DNA or RNA) can be detected in sufficient quantities and also the assay is highly sensitive and requires less incubation time. NAAT is widely used as a research tool for the identification and characterization of various viruses. The respiratory viral pathogens can be detected using various amplification techniques like PCR, RT-PCR, NASBA, strand displacement amplification and transcription mediated amplification. The performance of the NAAT assays depends on the site of sample collection and time; various procedures during extraction, amplification and detection techniques. A properly standardized and validated nucleic acid assays which are highly sensitive can be implemented in routine clinical practice for the early diagnosis of various respiratory viral pathogens (Leland and Ginocchio 2007). The advantage of molecular assays is that it has high sensitivity, less time consuming and also can be utilized to detect multiple viruses in one assay. The molecular methods have been reported to be more sensitive than the non molecular methods in practice (Freymuth et al., 2006; Kuypers et al., 2006; Kim et al., 2009; Sanghavi et al 2012). The major disadvantage of the NAAT is the high cost of the instruments and the reagents and chances of cross contamination (Mahony 2008; Loeffelholz and Chonmaitree 2010).

1.1.4.4 POLYMERASE CHAIN REACTION (PCR)
The polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single or a few copies of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. It was first developed by an American biochemist, Kary Mullis in the year 1984. It is a chain reaction (one DNA fragment is used to produce two copies, then four, eight and so on as per the requirement). It is accomplished with the help of polymerase enzyme (that join together the bases adenine, thymine, cytosine and guanine in proper sequence to form the particular strand of DNA), and primers (serve as template for the attachment of the bases). The technique selectively amplifies a particular small segment of the large DNA molecule. There are three major steps involved in PCR- denaturation,
annealing and extension. The DNA is denatured at high temperatures (between 90 - 97°C). In the second step, the primers anneal to the DNA strands to initiate extension followed by extension where the bases attach to the annealed primers to create a complementary strand. Each of the newly constructed DNA strand consists of one old strand and the new complementary strand. This cycle is repeated (30-35 times) to provide sufficient amount of the amplified cDNA product. The annealing of the primes occurs at a lower temperature (50-60°C) facilitating the hybridization of the primers to their respective complementary strands. The Taq polymerase adds the nucleotides to the end of the annealed primers. The extension step is carried out at approx 72°C for 2-5 minutes (Mullis & Faloona 1987; Saiki et al. 1988; Joshi and Deshpande, 2010). A reverse transcription reaction can also be performed for conversion of a RNA genome, followed by amplification of the cDNA as mentioned above. This can be done in a separate tube or as a single reaction, using a thermostable DNA polymerase with reverse transcriptase activity. Performing amplification in a single tube reduces the chances of cross contamination. Conventional PCR is a qualitative assay. The sensitivity of the conventional PCR assay can be increased by nucleic acid hybridization of the amplicon to a labelled oligonucleotide probe, targeted to a conserved sequence of the amplicon sensitivity. Hybridization can be done by liquid phase methods where it is performed using a microtiter plate to which the PCR product binds via a biotinylated primer. Further the specific enzyme conjugated probe and a substrate for the enzyme, and the reaction can be detected by spectrophotometer. It provides good sensitivity and specificity but is more laborious (Johnston et al. 1993; Freymuth et al. 1995).

1.1.4.5 REAL-TIME PCR

The real-time PCR allows the exact quantification of DNA/RNA with greater reproducibility. The advantages of real-time PCR over conventional PCR are the greater sensitivity, reproducibility and precision, quantification, lower risk of contamination and rapid analysis. The basic real-time PCR instrumentation consists of a thermal cycler with an optical system to detect the fluorescence and a computer with added software to perform the final analysis. The emission of the fluorescence generates a signal that is directly proportional to the amount of the amplified PCR product. The fluorescent substances commonly used are SYBR® Green and TaqMan® (Morillo et al. 2003; Novais et al. 2004; Kubista et al. 2006; Valones et al. 2009).
SYBR Green I is the most commonly used fluorescent dye. It fluoresces when bound to the dsDNA and the binding capacity is 100 times greater than that of ethidium bromide. Minor groove DNA binder probes (MGB) are 14 to 15bp long oligonucleotides that carry a fluorescent dye in the 5’ terminal and a non fluorescent quencher and MGB in the 3’ terminal that specifically bind to the target sequence. MGB is released from a probe that binds to the minor groove of the dsDNA (consisting of part of the MGB probe and complementary target sequence by which it is hybridized) related to the nucleotide sequence. The MGB increases binding stability to the amplification probe.

1.1.4.6 HYBRIDIZATION PROBES
Oligonucleotide probes marked with fluorophores are used for the detection of specific sequences. The amount of the fluorescence may be related to the amount of PCR product through the product dependent reduction of a quencher fluorophore and a reporter or through an increase in the fluorescent resonance energy transfer (FRET) from a donor fluorophore to a receptor. The donor probes are marked in the 3′ terminal portion with a reporter fluorophore and the acceptor probes are marked in the 5′ terminal portion with an acceptor fluorophore. Only the donor fluorophore is excited in such a way that no fluorescent acceptor is detected in the free-floating probes. During the annealing phase of the primer, the probes hybridize adjacent to the single-stranded DNA (ssDNA) and the excitation energy is transferred from the donor to the acceptor. Four oligonucleotides are used in this format: two primers and two probes. The amount of fluorescence is proportional to the amount of target DNA generated during the PCR process (Bernard and Wittwer, 2000; Valones et al. 2009). The various types of hybridization probes used are TaqMan probes, Molecular beacons, sunrise primers and scorpion primers.

Currently, PCR and its applications is an important tool in science and medicine, improving human health and life. PCR has completely revolutionized the detection of RNA and DNA viruses. PCR is valuable as a confirmatory test. PCR has also been recognized to have been able to detect mixed infections with ease in many studies. PCR is a rapid technique with high sensitivity and specificity, a more sophisticated technique but requires infrastructural support and is expensive when compared to the conventional diagnostic methods.
1.1.5. HUMAN METAPNEUMOVIRUS
1.1.5.1 DISCOVERY AND CLASSIFICATION

Human metapneumovirus (hMPV) was initially isolated in The Netherlands from 28 nasopharyngeal aspirates (NPA) collected from children less than 5 years of age presenting with respiratory tract infections over a 20 year period. The virus replicated very slowly in tertiary monkey kidney cells and the cytopathic effect produced was similar to that of RSV. On electron microscopy of the supernatant from the infected cells presence of paramyxovirus like pleomorphic particles with a diameter of 150 to 600nm with short projections of 13 to 17nm length were observed (Figure 1).

Fig: 1 Electron micrograph of hMPV (Peret et al. 2002)

The nucleocapsid was not visible as in the case of certain other Paramyxoviruses like RSV and parainfluenza. It was inactivated by chloroform and did not agglutinate erythrocytes. Reverse transcriptase reaction using primers specific for other respiratory viruses also did not produce any positive results. Based on the genomic pattern and morphological features it was classified under the Paramyxoviridae, subfamily Pneumovirinae and genus Metapneumovirus. The other pathogenic species in this subfamily known is the Avian pneumovirus (van den Hoogen et al. 2001) (Figure 2).
1.1.5.2 STRUCTURE
The virion of hMPV is spherical, filamentous or pleomorphic and consists of a helical nucleocapsid enclosed within a lipid bilayer envelope derived from the plasma membrane of the host cell. The three transmembrane surface glycoproteins F, G and SH are embedded in this envelope. They appear as small spikes projecting from the surface of envelope. The spikes are approximately 15nm in length and are associated with attachment and entry of the virion into host cell (van den Hoogen et al. 2001; Peret et al. 2002).

1.1.5.3 GENOMIC ORGANIZATION
The negative sense single stranded RNA genome of hMPV is approximately 13000 nucleotides in length. It is encapsidated with the N protein and contains eight genes in the order 3’-N-P-M-F-M2-SH-G-L-5’ which encode for nine different proteins and is similar to the RSV and AMPV (van den Hoogen et al. 2002; Biacchesi et al. 2003) (Figure 3). The proteins in hMPV are N, nucleoprotein; P, phosphoprotein; M, matrix protein; F, fusion protein; M2-1, product of the first open reading frame (ORF) in the M2 mRNA; M2-2, product of the second ORF in the M2 mRNA; SH, small hydrophobic glycoprotein of unknown function; G, attachment glycoprotein; and L, viral polymerase. The proteins and their function are analogous to that of RSV except that the NS1 and NS2 are lacking in hMPV (Biacchesi et al. 2005). The N protein is bound to the RNA genome and forms the ribonuclease (RNase) resistant nucleocapsid core. The phosphoprotein (P) and L protein are attached to this core in clusters. This complex of proteins called the ribonucleocapsid complex (RNP) has RNA-dependent RNA transcriptase activity and initiates intracellular virus replication (Lamb and
Parks, 2007). In addition, the genome contains non-coding 3’ leader and 5’ trailer sequences and intergenic regions (Herfst et al. 2004). The viral promoter is contained within the 3’-terminal 57 nucleotide of the genome (Biacchesi et al. 2004a). The hydrophobic viral M protein located in between the envelope and the core is important in virion structural design and is released from the core during virus entry (Lamb and Parks, 2007). The M2 gene is present in all members of the subfamily Pneumovirinae (van den Hoogen et al. 2002). The M2 gene of hMPV contains two overlapping ORFs that encode 2 proteins, M2-1, and M2-2 (Kitagawa et al. 2010). The M2-1 ORF is not essential for replication in hMPV unlike as in the case of RSV where the M2-1 gene is required for the viral transcription and replication (Buchholz et al. 2005). M2-2 gene expressed protein is functional in regulation of RNA synthesis, preserving the genetic stability of the hMPV genome and can be considered as a multifunctional protein (Buchholz et al. 2005; Kitagawa et al. 2010; Schickli et al. 2008).

**Fig. 3: Comparative genomic organization of hMPV and RSV (Schildgen et al.)**

1.1.5.3.1 THE NUCLEOPROTEIN (N GENE)

The nucleoprotein forms an integral part of the nucleocapsid of the virion and is tightly associated to the RNA genome and gives the RNA genome its helical structure (Easton et al. 2004). The RNA genome in association with the N, P and L protein forms the highly stable, RNase resistant RNP complex. The N protein is essential for recognition by the viral RNA dependent RNA polymerase, necessary for both transcription and replication (Melero, 2007).

The N terminal end of the N protein is highly conserved among members of the *Mononegaviridae*. The N gene of hMPV, 1185 nucleotides (nt. 55–1239) in length (van den Hoogen et al. 2001), encodes a 394 amino acid protein and has a molecular weight of 43.5KDa. It shares an 88% amino acid sequence identity with APV-C and
41% amino acid identity with RSV (Biacchesi et al. 2003, 2005). Three conserved regions (between amino acids 160-172; 251-263 and 278-327) in the N protein present among members of the family Paramyxoviridae are also present in hMPV (Biacchesi et al. 2005).

1.1.5.3.2 THE PHOSPHOPROTEIN (P GENE)

The phosphoprotein of pneumoviruses attached to the RNA genome is part of the nucleocapsid and is considered to be involved in both replication and transcription (Easton et al. 2004). The hMPV P protein in combination with the N protein is the minimal requirement for the formation of inclusion like complexes in the mammalian cells (Derdowski et al. 2008). The hMPV phosphoprotein contains an ORF of 294 amino acids (884 nucleotides- no: 1263 to 2147) and has a molecular mass of 32.5KDa like other pneumoviruses. The ORF lacks cysteine residues and is rich in glutamine at the C terminus (van den Hoogen et al. 2002; Bastien et al. 2003; Biacchesi et al. 2003). It shares 68% amino acid identity with the P protein of APV-C and only 22 – 24% with the P protein of RSV. The highly conserved region is between amino acids 185-241 and is involved in synthesis of RNA and maintaining the integrity of the nucleocapsid (van den Hoogen et al. 2002). The nucleotide sequence identity, with respect to the P protein of hMPV, is more (91-100%) among the members of the same sub-group when compared to the identity (78-79%) between the sub groups, the majority of the substitutions being in the amino proximal terminal of the protein (Bastien et al. 2003; Ishiguro et al. 2004).

1.1.5.3.3 THE MATRIX (M) PROTEIN

The hMPV M protein is present on the inner surface of the lipid envelope and forms the link between the nucleocapsid and the envelope (Easton et al. 2004). It has a molecular mass of 27.6KDa and the ORF encodes a 254 amino acid protein that is similar to the M proteins of other pneumoviruses. It has a high amino acid sequence identity with the matrix protein of AMPV (76-87%) and only has low amino acid sequence identity with RSV (37-38%) (Bastien et al. 2003; van den Hoogen et al. 2002). The sequence identity of the M gene of hMPV within the sub groups and among the subgroups is 94-100% and 83-85% respectively (van den Hoogen et al. 2002; Bastien et al. 2003; Biacchesi et al. 2003; Ishiguro et al. 2004). There are two secondary ORFs, one small ORF of 54 amino acid residues (nt. position 2281) within
the major M ORF and the other one overlapping the major M ORF of 33 amino acid residues (nt. position 2893) (van den Hoogen et al. 2002). The genes encoding the M2 proteins contain two overlapping ORFs M2-1 and M2-2, are unique for all pneumoviruses, and functions as a transcription elongation factor in RNA synthesis (Easton et al. 2004).

The M2-1 is involved in virus RNA synthesis, functioning as a transcription elongation factor (Easton et al. 2004). The M2-1 protein in association with the N and P proteins forms the inclusion bodies (Collins and Crowe, 2007). It enhances the processivity of the viral polymerase and has anti-termination activities which are essential for the synthesis of complete mRNA transcript (Collins et al. 1996). The N terminus is considered the most conserved region in the M2-1 gene. It contains three cysteine residues in the first 30 amino acids, which is common in zinc binding proteins, and the Cys/His motif in M2-1 gene of all pneumoviruses may have functional importance (van den Hoogen et al. 2002; Collins and Crowe, 2007). The M2-1 ORF of hMPV encodes for the M2-1 protein of 187 amino acid residues. It is located near the F gene has 84% identity with M2-1 of APV and only and 35-36% identity with RSV. The N terminus of the M2-1 gene of hMPV is highly conserved and exhibit 100% similarity with APV-C in the first 80 amino acid residues of the protein, which is in accord to that of all members of the pneumoviruses. It also consists of three cysteine residues in the first 30 amino acid residues which are conserved among all pneumoviruses (van den Hoogen et al. 2002).

The second ORF (M2-2) is conserved in pneumoviruses overlaps with the M2-1 ORF. It mediates the switchover from transcription to replication in viral RNA synthesis. The M2-2 ORF is 77 amino acids in length, and starts at the 512 nucleotide in the M2-1 ORF which is same for APV-C. The amino acid sequence identity between the M2-2 ORF of hMPV and APV-C is 56% (van den Hoogen et al. 2002).

1.1.5.3.4 THE SMALL HYDROPHOBIC (SH) PROTEIN
The SH protein of hMPV is a type II transmembrane glycoprotein placed near the amino terminus of the plasma membrane and inserted by a hydrophobic signal-anchor sequence. The SH gene located near the M2 gene is made up of 183 amino acid
residues and is the largest SH protein among the pneumovirus subfamily. The region is poorly conserved between sub-groups (as in other pneumoviruses) having a nucleotide sequence identity of 69% and a higher percentage identity of 91% between members of the same sub-group (Biacchesi et al. 2004b; Ishiguro et al. 2004; van den Hoogen et al. 2002; Skiadopoulos et al. 2006). The SH ORF has a high composition of threonine and serine residues and has a similar hydrophilicity to that of the RSV and AMPV SH protein. The SH ORF of the hMPV has a hydrophilic N-terminus, a central hydrophobic domain, a second hydrophobic domain, and a hydrophilic C-terminus similar to the SH ORF of AMPV, which is lacking in the SH ORF of RSV (van den Hoogen et al. 2002). The SH protein of hMPV exists in multiple forms and with altering lengths -SH0, SHg1and SHg2 (Biacchesi et al. 2003; Ishiguro et al. 2004). Experimental studies using recombinant mutants of hMPV suggest that the SH protein has no specific role in the viral attachment and entry. The antibodies produced against SH protein also has no role significant effect in neutralization or protection of the host protein (Biacchesi et al. 2004b; Buchholz et al., 2005; Skiadopoulos et al.2006). Absence of SH protein may enhance in vivo and invitro secretion of IL-6 and IL-8 (Bao et al. 2007).

1.1.5.3.5 THE ATTACHMENT GLYCOPROTEIN (G)

The hMPV G protein is a type II transmembrane glycoprotein that mediates attachment during viral infection (van den Hoogen et al. 2002; Biacchesi et al. 2003; Biacchesi et al. 2004b; Ishiguro et al. 2004; Peret et al. 2004; Skiadopoulos et al. 2006). The G ORF encodes a 236 amino acid protein and is adjacent to the SH gene (nt6262–6972). There are four small ORFs in the G gene all of which lack a start codon (van den Hoogen et al. 2002). The length of the amino acids may vary from 217 to 236 due to the usage of four different transcription termination codons and also due to the nucleotide substitutions situated in the extracellular domain. The nucleotide identity between the subgroups is 58% and between the members of the subgroups it is 76%, thereby exhibiting high sequence. The G protein is highly glycosylated like other mucinogenic glycoproteins and has high serine-threonine and proline content (Biacchesi et al. 2003; Bastien et al. 2004; Ishiguro et al. 2004; Peret et al. 2004; van den Hoogen et al. 2004; Ludewick et al. 2005; Galiano et al. 2006). The serine-threonine and proline content is more in hMPV than RSV and APV. There are five N linked glycosylation sites in the G ORF of hMPV. The hydrophobicity of the G ORF
of hMPV is similar to that of other pneumoviruses. The N terminus has a hydrophilic region followed by short hydrophobic area and hydrophilic C terminus similar to that of RSV and APV. There is only one cysteine residue in the G ORF of hMPV when compared to five in RSV and 20 in APV. Among all the four secondary ORFs there is only one cysteine residue, but it contains 12–20% serine and threonine residues and 6–11% proline residues (van den Hoogen et al. 2002).

1.1.5.3.6 THE FUSION F PROTEIN

The amino acid sequence of hMPV F protein has 81% identity with AMPV-C, 67% with AMPV-A and -B, 33–38% with other pneumovirus F proteins, and only 10–18% with other members of the Paramyxoviridae (van den Hoogen et al. 2002). Inspite of the low amino acid sequence identity, the structure (type I integral protein) and function of the hMPV F protein is similar to that of other pneumoviruses (van den Hoogen et al. 2002; Biacchesi et al. 2006). The hMPV F glycoprotein is 539 amino acids in length and has a molecular mass of 58.4 KDa (van den Hoogen et al. 2002; Bastien et al. 2003; Biacchesi et al. 2004b; Biacchesi et al. 2006; Schowalter et al. 2006). It is glycosylated containing three potential N glycosylation sites. Like other paramyxoviruses, the F protein of hMPV is synthesized as an inactive precursor which is cleaved by the host cell proteases into two fragments (F1 and F2), which remain attached by the disulphide bond. The 23 amino acid F1subunit contain the fusion peptide at its N terminal (Biacchesi et al. 2006). The hMPV F protein has only one cleavage site (between residues 114 and 115) containing the RQSR residues whereas RSV contains two cleavage sites one with RQSR and the other with RARR residues. The three hydrophobic domains of the primary F protein sequence are the signal located at the amino acid terminus of F2 of the subunit, the fusion and membrane anchor domains located in the F1 subunit at N; and the C termini respectively. There are two domains present in the F protein namely HRA and HRB. They are located in the F1 subunit and are essential for the viral fusion. The HRA located adjacent to the fusion domain and HRB adjacent to the membrane anchor domain at the carboxy terminal (van den Hoogen et al. 2002).

1.1.5.3.7 LARGE POLYMERASE PROTEIN (L)

The L protein of the pneumoviruses is thought to be the major component of the viral RNA-dependent RNA polymerase complex involved in the synthesis of all viral RNA
(van den Hoogen et al. 2002; Easton et al. 2004; Collins and Crowe, 2007). It is involved in the enzymatic processes such as methylation, capping and polyadenylation. All the six domains present in other negative sense RNA viruses has been identified in hMPV L protein among which four core polymerase motifs are important in polymerase function (van den Hoogen et al. 2002). The L gene of hMPV encodes a 2005 amino acid protein, and shares a sequence identity of 64% with APV-A, 44% with RSV, and 13–15% with other paramyxoviruses. HMPV L proteins within virus strains of the same subgroup have a nucleotide sequence homology of 95% compared with 84% homology between subgroups (van den Hoogen et al., 2002; Biacchesi et al., 2003).

1.1.5.4 REPLICATION OF THE VIRUS

The replication of hMPV is initiated by the attachment of the virus to the host cell mediated by the G protein. The hydrophobic protein present near the N terminus serves as the uncleaved signal peptide and the membrane anchor. The high content of serine-threonine and proline residues in the G protein facilitates the attachment of the virus to the host cell. The cellular glycosaminoglycans like heparin sulphate are involved in the binding of the G protein to the host cell (Thammawat et al. 2008). In vitro studies using recombinant viruses lacking attachment glycoprotein and in vivo studies in African green monkey (Biacchesi et al. 2005) has demonstrated the entry and proliferation of the virus, suggests that the G protein may not be a necessary factor for the entry of hMPV into the host cell. The F protein facilitates the fusion of the virus to the host cell membrane. It is a type I membrane protein similar to other pneumoviruses. It is synthesized as an inactive precursor F0 which is cleaved to active subunits F1 and F2. The in vivo cleavage of the F protein in hMPV usually occurs at neutral pH requiring exogenous protease activity and the in vitro cleavage is facilitated by addition of trypsin (Schildgen et al. 2011). The hMPV F protein binds with $\propto$v$\beta$1conserved Arg-Gly-Asp motif for attachment to the host cell in the absence of G protein (Cseke et al. 2009). The fusion of the hMPV F protein is triggered by low pH (Schowalter et al. 2006) and the triggering process is contributed by electrostatic repulsion in the heptad repeat B linker region. Recent studies have suggested that the hMPV entry inside the host cell is by endocytosis. The low pH environment after the entry of the virus may play an important role in triggering the fusion mechanism of the virus (Schowalter et al. 2009). Following the membrane
fusion the helical nucleocapsid containing the viral RNA is released into the host cell. Uncoating occurs and the negative sense viral RNA is released into the cytoplasm, which serves as the template for the synthesis of the mRNA and the antigenomic cRNA. The genomic RNA synthesized by the antigenome produces additional antigenomes to be incorporated into the daughter virion or it is used as template for secondary transcription. Once translation is complete the ribonucleoproteins and the M proteins are synthesized in the cytoplasm, and the surface glycoproteins F, SH, G synthesized in the endoplasmic reticulum are transported to and organized in selected regions in the plasma membrane. The M proteins play a major part in the viral assembly. They act as adapters occupying a central position that link RNP at the core and the viral glycoproteins at the exterior. The virion assembly occurs in the plasma membrane and the progeny virions are released by budding (Lamb and Parks, 2007; Schildgen et al. 2011) (Figure 4).

Fig. 4: Schematic representation of hMPV life cycle (Schildgen et al. 2011)

1.1.5.5 TRANSMISSION AND CLINICAL MANIFESTATIONS
Human metapneumovirus is associated with ARI in all age groups, the infection being more predominant in infants and young children (Bastein et al. 2003; Williams et al. 2004). The infection is also seen in elderly, immunocompromised and is also a cause of nosocomial infection in paediatric wards (Falsey et al. 2003; Mahalingam et al., 2006; van den Hoogen et al. 2007). Like other respiratory viruses hMPV also spread
through close contact with patients or fomities. The incubation period is approximately one week. hMPV mainly infect the airway epithelial cells causing degeneration and necrosis. The clinical manifestations of hMPV infection are similar to that of RSV which includes mild upper respiratory infections to severe infections like bronchiolitis, wheezing as well as pneumonia. The obstruction of the bronchioles and the alveoli associated with and hMPV infections may be due to the presence of increased mucous, mononuclear cells and cell debris (Vargas et al., 2004; Collins and Crowe, 2007). It is also considered as a cause of acute wheezing in children and may trigger asthma (Peiris et al., 2003; van den Hoogen et al., 2003; Williams et al., 2004; Williams et al., 2005; Agapov et al., 2006; Vicente et al., 2006; Manoha et al., 2007).

1.1.5.6 MOLECULAR EPIDEMIOLOGY OF hMPV
Two genetic lineages (A and B) of hMPV were described by van den Hoogen et al. as soon as the virus was discovered by the phylogenetic analysis of the partial nucleotide sequences of the N, F, M and L ORFs (van den Hoogen et al. 2001) and two sub groups were also identified by further phylogenetic studies (van den Hoogen et al. 2001; Peret et al. 2002; Boivin et al. 2002; Viazov et al. 2003; Maggi et al. 2003; Biacchesi et al. 2003; Bastien et al. 2003a; Bastien et al. 2003b; Ebihara et al. 2004b; Ishiguro et al. 2004). The amino acid sequence identity of F protein among A and B groups of the viruses was found to be maximum (95 to 97%) and that of the G protein was minimum (30 to35%). The world wide prevalence of the different genetic lineages have been established and it proves that a particular lineage is not restricted to a particular place and season and also multiple lineages can co circulate in the same period or season at a specific area or location (Schildgen et al. 2011). Two sub groups of A2 lineage (A2a and A2b) have been reported and the A2 sub lineage shows maximum diversity (Huck et al. 2006). The clinical severity of the infection by hMPV based on the genetic lineage is a controversial issue. Certain studies have reported that the lineage A is more prevalent and causes clinically severe respiratory tract infections (Martinello 2002; Kaida et al. 2006; Vicente et al. 2006; Arnott et al. 2012) and some reports suggest that the infection by the B sublineage causes more severe infections than the A lineage (Esper et al. 2004; Pitoiset et al. 2010). There are reports that there is no proof regarding the association of the clinical severity of hMPV infection and genetic lineage (Agapov et al. 2006; Larcher et al. 2008; Xiao et al. 2010).
1.1.5.7 GEOGRAPHICAL AND SEASONAL DISTRIBUTION

Since the discovery of hMPV in 2001 it has been emerging as a major cause of respiratory tract infections, especially in children, throughout the world. The prevalence has been reported from all the continents- Middle East (Regev et al. 2006; Kaplan et al. 2008), Africa (El Sayed Zaki et al. 2009; Berkley et al. 2010), The Caribbean (Matthew et al. 2009), North America (Boivin et al. 2002; Esper et al. 2003), Central America (Noyola et al. 2005; Ulloa-Gutierrez et al. 2009), South America (Galiano et al. 2004; Gray et al. 2006; Escobar et al. 2009; Pizzorno et al. 2010), Australasia (Nissen et al. 2002; Peiris et al. 2003; Thanasugarn et al. 2003; Werno et al. 2004; Rao et al. 2004; Ebihara et al. 2004b; Kim and Lee, 2005; Lin et al. 2005; Abdullah Brooks et al. 2007; Loo et al. 2007; Li et al. 2009; Chen et al. 2010; Xiao et al. 2010; Do et al. 2011; Arnott et al. 2013), United Kingdom & Europe (van den Hoogen et al. 2001; Jartti et al. 2002; Stockton et al. 2002; Christensen et al. 2003; Maggi et al. 2003; Viazov et al. 2003; von Linstow et al. 2004; Xepapadaki et al. 2004; Carr et al. 2005; García-García et al. 2006; Larcher et al. 2006; Sivaprasakam et al. 2007; Tecu et al. 2007; Baer et al. 2008; Ljubin-Sternak et al. 2006; Rafiefard et al. 2008; Pavlova et al. 2009; Antunes et al. 2010; Legrand et al. 2011). The infection rate is at its peak in the late winter and early spring in temperate regions of the world (Esper et al. 2004; Williams et al. 2004; Agapov et al. 2006). hMPV infections has also been observed in low frequencies during autumn and summer (van den Hoogen et al. 2003; Williams et al. 2004; Esper et al. 2004; McAdam et al. 2004; Mullins et al. 2004; Chano et al. 2005; Louie et al. 2007; Oliveira et al. 2009;). There are reports regarding the prevalence of hMPV infection in sub tropical regions in spring and early summer (Peiris et al. 2003; Wang et al., 2008), which indicates that the hMPV circulates throughout the year.

1.1.5.8 DIAGNOSIS

The diagnosis of hMPV infection can be approached using various techniques like cell culture, nucleic acid amplification tests, antigen detection and serological methods. HMPV replicates poorly in conventional cell cultures and reveals mild cytopathic effects. Also the technique is laborious, expensive and requires special procedures like trypsin addition. The various cell lines in which hMPV can be cultivated are tertiary monkey kidney cells, Vero cells, LLC-MK2-cells, BEAS-2B cells, A549 cells, and HepG2. The cytopathic effects are seen in tertiary monkey
kidney, LLC-MK2 and Vero cells lines but only after 10 to 21 days of incubation (van den Hoogen et al. 2001, Bao et al 2007, Schildgen et al. 2010, Tollefson et al. 2010). Shell vial culture technique which includes centrifugation, short incubation and fluorescent staining is a rapid method for the identification of hMPV (Landry et al. 2005). Direct immunofluorescence assay is a rapid method for the identification of hMPV in which labelled antibodies are used for the identification of hMPV antigen in respiratory specimens. ELISA and microarray methods are also used but are not available commercially. Reverse transcriptase PCR assays amplifying the viral RNA is the most widely used and sensitive method employed for the identification of hMPV. Various regions viz. F, N, G, L, M are commonly used as targets, among which the F and N gene are considered to be more specific and conserved, suitable for identification of hMPV.

1.1.5.9 TREATMENT
Ribavirin has a broad spectrum inhibitory activity against both DNA and RNA viruses. Treatment with oral ribavirin has proved effective against hMPV both in vitro and in vivo (Shachor-Meyouhas et al. 2011). Palivizumab and Motavizumab are monoclonal antibody preparations specific for RSV which are also effective against hMPV (Feltes et al. 2003, Carbonell-Estrany et al. 2010). The MAb338 which targets the hMPV fusion protein has shown good results in animal models (Ulbrandt et al. 2006). Human Fab DS7 has demonstrated therapeuetic and prophylactic activity both in vivo and in vitro (Williams et al. 2007). The recent therapy for hMPV is based on fusion inhibitors and RNA interference.

1.1.6 HUMAN BOCAVIRUS
1.1.6.1 DISCOVERY AND CLASSIFICATION
Human Bocavirus (HBoV) was discovered in Sweden, in the year 2005 from the pooled cell free filtrates of the NPA from children with ARI by molecular screening methods. HBoV belongs to the family Parvoviridae, subfamily parovirinae and genus Bocavirus. The genus Bocavirus consists of bovine parovirus (BPV), canine minute virus (CMV) and human Bocavirus (HBoV) (Allander et al. 2005). At present there are four subtypes of HBoV (1 to 4) have been identified. HBoV 1 is the common subtype found in respiratory specimens whereas the other three are
commonly identified in gastrointestinal specimens (Volz et al. 2007; Schildgen et al. 2008; Schildgen et al. 2011; Kapoor et al. 2010).

1.1.6.2 STRUCTURE
HBoV belongs to the Parvoviridae family and are small non- enveloped. The nucleocapsid is 18 to 26nm in size and contains a single stranded negative sense DNA as the genome (Allander et al. 2005; Allander et al. 2007; Böhmer et al. 2009; Schildgen et al. 2012) (Figure 5).

**Fig. 5: Diagramatic representation of HBoV virus (Gurda et al. 2010)**

1.1.6.3 GENOMIC ORGANIZATION
The length of the single stranded negative sense DNA of HBoV is 5,217 to 5,299 nucleotides. In addition there are 32 to 52 nucleotides attached, forms the terminal sequences, which plays an important role in the replication of viruses in Parvoviridae family (Lusebrink et al. 2011; Kapoor et al. 2011).

The HBoV genome consists of three open reading frames (ORFs) namely first one encoding the non structural protein, NSI, located at the 5’ end followed by the second ORF which encodes another non structural protein NP1, unique for HBoV. The last ORF at the 3’ end encodes two viral capsid proteins, VP1 and VP2. The viral capsid protein of HBoV has approximately 40% amino acid sequence similarity with that of BPV and CMV (Allander et al. 2005). The function of HBoV NS1 and NP1 is unknown. These proteins in other viruses of the parvovirinae subfamily have multifactorial functions such as replication, cell cycle arrest, apoptosis etc (Sol et al 1999; Christensen et al.1995; Fu et al. 2002; Nakashima et al. 2004; Hsu et al. 2006; Sun et al. 2009;) The NS1 and NP1 regions are proved to be more conserved than the VP1 and VP2 regions (Chieochansin et al. 2008) (Figure 6).
1.1.6.4 REPLICATION OF THE VIRUS

The paroviruses generally replicate by the rolling hair pin mechanism. In this mechanism the DNA fragments are obtained by the copying the coding sequences and the termini twice either in the head to head or tail to tail manner (Tattersall et al. 1973; Cotmore and Tattersall 1984). The presence of head to tail arranged sequence hypothesized the presence of another alternative method of replication in HBoV ie. the rolling circular model (Lusebrink et al. 2011; Kapoor et al. 2011) (Figure 7).

1.1.6.5 TRANSMISSION AND PATHOGENESIS

The exact mode of transmission of HBoV infection is unknown. HBoV1 is presumed to be transmitted through inhalation aerosols contaminated by the virus similar to the mode of transmission of other paroviruses. HBoV has been detected in urine, faeces and is also known to cause viraemia in the active stage of replication in the host (Kantola et al. 2008; Hedman et al. 2010; Don et al. 2010; Korppi et al. 2010; Don et
al. 2011; Kantola et al. 2011; Korner et al. 2011; Nascimento-Carvalho 2012). Nosocomial acquirement of HBoV infection has also been reported (Kesebir et al. 2006; Chow et al. 2008; Calvo et al. 2008; Zeng et al. 2010; Durigon et al. 2010).

The pathogenesis of HBoV is not well established due to the lack of standardized in vitro culture methods and animal models. There are studies across the world which suggests that HBoV is a respiratory pathogen. It is known to cause lower respiratory tract infection in and the symptoms associated are acute wheezing, bronchiolitis, fever and pneumonia (Allander et al. 2007; Fry et al. 2007; Terrosi et al. 2007; Zhang et al. 2008; Calvo et al. 2008; Smuts et al. 2008; Dina et al. 2009; García-García et al. 2010; Antunes et al. 2010). The occurrence of HBoV infection in immunocompromised (transplant) individuals has also been established (Schenk et al. 2010). HBoV DNA has been detected in serum of patients with acute primary and severe infection (Kantola et al. 2008; Christensen et al. 2010; Don et al. 2010). There are several studies that indicate the role of HBoV (especially subtypes 2, 3 and 4) as a gastrointestinal pathogen (Vicente et al. 2007; Szomor et al. 2009). HBoV has also been identified from urine specimen (Pozo et al. 2007). The pathogenicity of HBoV can be assumed to be analogous to that of minute virus of canines (MVC). The virus enters the body through the respiratory tract, multiplies enters the blood stream and finally the gastrointestinal tract through blood or by ingestion. The viral shedding occurs either by coughing or by defecation (Tijssen 1999). There are reports suggesting latent infection or the persistence of HBoV in patients (Longtin et al. 2008; Lu et al. 2008). There is a high rate of co-infection of HBoV with other viruses (Christensen et al. 2008; Gagliardi et al. 2009; Garcia et al. 2010).

1.1.6.6. CLINICAL MANIFESTATIONS
The clinical manifestations of HBoV infections are indistinguishable from that of other respiratory pathogens (Pavia 2011). The common respiratory symptoms seen in HBoV infected individuals are wheezing, respiratory distress, fever, cough, rhinorrhea, bronchiolitis and pneumonia (Arnold et al. 2006; Choi et al. 2006; Kesebir et al. 2006; Calvo et al. 2008; Blessing et al. 2009). HBoV 1 has been identified in the NPA and middle ear fluid from children with acute otitis media (Ruohola et al. 2006; Kleines et al. 2007; Beder et al. 2009). Several studies have detected HBoV in stool samples of children with acute gastrointestinal disorder but its pathogenicity is
uncertain (Vicente et al. 2007; Albuquerque et al. 2007; Lau et al. 2007; Lee et al. 2007). The study by Arthur et al. (2009) has reported the association of HBoV 2 with gastroenteritis. The risk factors associated with HBoV infection is similar to those for other respiratory viruses like congenital heart diseases, asthma, chronic obstructive pulmonary, immunosuppression, maternal smoking, premature birth and winter birth time. Day care centers and drinking of sewage or river water may also be a factor for HBoV infection (Hamza et al. 2009; Blinkova et al. 2009; Räsänen et al. 2010).

1.1.6.7 EPIDEMIOLOGY

HBoV is a pathogen of the respiratory and gastrointestinal tract. After its initial discovery in Sweden in 2005, it has been identified from around the globe in all continents- Europe (Weissbrich et al. 2006; Allander et al. 2007; Volz et al. 2007; Kleines et al. 2007; Terrosi et al. 2007; Garcia-Garcia et al. 2008; Bonzel et al. 2008; Fabbiani et al. 2009; Soderlund-Venermo et al. 2009; Modrow et al. 2011), North America (Bastien et al. 2006; Longtin et al. 2008; Albuquerque et al. 2009) South America (Flores et al. 2011; Salmon-Mulanovich et al. 2011; Pilger et al. 2011; Ghietto et al. 2012; Ghietto et al. 2012), Africa (Smuts et al. 2008), Asia (Lau et al. 2007; Chieochansin et al. 2008; Pham et al. 2011; Khamrin et al. 2012), and Australia (Arden et al. 2006; Sloots et al. 2006; Arthur et al. 2009; Tozer et al. 2009; Arden et al. 2010). There are subtypes of HBoV identified namely HBoV1, 2, 3 and 4. HBoV1 is more frequently associated with respiratory tract infections whereas the other three subtypes are commonly associated with gastrointestinal disorders. The prevalence of HBoV1 is 2 to 19% and more commonly seen in children between the age group of 6 to 24 months. HBoV1 infection is more predominant in the winter and spring (Allander et al. 2005; Arnold et al. 2006; Fry et al. 2007; Cheng et al. 2008; Do et al. 2011; Arnott et al. 2013). HBoV1 has also been detected in adults (Bastien et al. 2006; Costa et al. 2009; Garbino et al. 2009). Among the enteric subtypes HBoV 2 is more prevalent (21 to 26%) (Kapoor et al. 2010) followed by HBoV3 (Cashman et al. 2012) and HBoV4 (Koseki et al. 2012). The seroepidemiology of HBoV is age related. The seropositivity of HBoV IgG antibodies is approximately 40% in the age group of 18 to 24 months and is 100% in children above 2 years of age (Kantola et al. 2011; Hustedt et al. 2012).
1.1.6.8 DIAGNOSIS
Currently, there is no adequate culture method developed for identification of HBoV. Hence the identification of the virus is commonly carried out from NPAs using conventional and real time PCR assays mostly targeting the NS1, NP1 and VP1/2 genes (Schildgen et al. 2008). There are other molecular methods also for the detection of HBoV (Bohmer et al. 2009). The real time PCR is more sensitive, specific and time saving when compared to conventional PCR assay methods. A wide range of commercial multiplex assay methods are also available for the detection of HBoV (De Vos et al. 2009, Balada-Llasat et al. 2011). IgG avidity EIA has been developed for the accurate diagnosis of primary infection and immunoactivation of HBoV infection (Hedman et al. 2010). Detection of antibodies against HBoV in serum can be carried out using ELISA methods with virus like particles (VLPs) of VP1 and VP2 (Kahn et al. 2006; Lin et al. 2008; Cecchini et al. 2009). Immunofluorescence assays for the detection of IgG antibodies and assays based on biomarkers are encouraging methods for the identification of HBoV infections (Shirkoohi et al. 2010; Sumino et al. 2010; Kumar et al. 2010). Initial screening of clinical samples (respiratory or stool) followed by the subsequent serum sample will help in the accurate diagnosis of HBoV infection as the virus will be present in the blood during the active infective stage.

1.1.6.9 TREATMENT
No specific in vivo or invitro antiviral therapy or prevention by immunization is available for HBoV. There are only supportive measures present. The transmission of the virus through contaminated aerosols should be prevented by using standard precautions.