Human Metapneumovirus in infants and young children in Thailand with lower respiratory tract infections; molecular characteristics and clinical presentations.

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Abstract

Two hundred and thirty six nasopharyngeal secretions (NPs) were lower respiratory tract infection collected from infants and children presenting with ALRI at Chulalongkorn hospital between March 2001 and September 2003 with 220 samples found eligible for analysis. We employed reverse transcriptase-polymerase chain reaction (RT-PCR) applying specific primers to identify hMPV and hRSV and also performed phylogenetic analysis of hMPV N, F and L. Of the 220 infants and young children tested, positive results were found in 12 (5.4%) and 118 (54%) specimens for hMPV and hRSV, respectively. The mean age of children with hMPV infections was 22±11 months. They mostly presented with fever with cough (100%) and upper respiratory tract symptoms (83%). Eleven of twelve infants were hospitalized. Additionally, phylogenetic analysis identified 2 distinct lineages of hMPV. Our results demonstrated the incidence of hMPV at 5.4% although the incidence in Thailand may be higher, as asymptomatic individuals were not tested.

Article Summary line

Human metapneumovirus was a new respiratory tract virus and accounted for significant morbidity and mortality especially in young children worldwide. We demonstrated the incidence, characterization and clinical aspects of hMPV infection in Thailand.

Introduction.

Respiratory virus infections account for significant morbidity and mortality especially in young children worldwide.[1-3] The viruses most frequently associated with respiratory tract infections include respiratory syncytial virus (RSV), parainfluenza viruses, adeno-virus and influenza virus.[4] Due to the difficulty in distinguishing between respiratory pathogens clinically and by laboratory based analysis, studies on the impact of respiratory infections are still quite limited. Despite improved sensitivity of diagnostic techniques, the cause of a significant portion of lower respiratory tract infections has still eluded identification.[5-7]

In 2001, Van den Hoogens et al.[8] were the first to describe a new respiratory virus, named human metapneumovirus.(hMPV). It has been classified as a member of the *Paramyxoviridae* family, genus *Pneumovirinae*.[8,9] Earlier discovery of hMPV had been delayed until recently as owing to its slow growth the virus has been difficult to detect in cell culture making the arrival of better molecular techniques such as RT-PCR imperative [10]. This provided an opportunity to define the extent of disease caused by a previously unknown pathogen. Subsequently, hMPV was detected in several countries as for example Canada,[11] France,[1] Italy[12] and USA.[13] It is likely to exist on a global scale. In Thailand, we recently identified hMPV and reported our preliminary results.[14] In the present study, our aim has been to further analyze and identify the molecular characteristics of this virus in conjunction with clinical presentations, information that will prove important for future development of specific antiviral therapies and vaccines.

Materials and methods.

Study population

We studied nasopharyngeal secretions(NPs) obtained from infants and young children with lower respiratory tract illness attending either the out-patient clinic or admitted to King Chulalongkorn Memorial hospital between March 2001 and November 2003. The study protocol was approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University. Parents were informed as to the study objective and their written consent was obtained before specimen collection. All the specimens were kept at -70 ºC until further analysis.

hMPV detection and characterization

A total of 220 stored NPs were thus analyzed by RT-PCR using primers specific for the Nucleoprotein(N), Fusion (F) and Polymerase(L) genes, respectively. RNA extraction was performed with the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA pellet was resuspended in 30-50 µl diethylpyrocarbonate-treated sterile water (DEPC water) and directly used as a template for cDNA synthesis. The RNA template was added to 20 µl cDNA reaction mixture containing 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT, 10 mM dNTP, 20 U RNAse inhibitor (Promega, WI), 25 U multireverse transcriptase (Promega, WI) and outer reverse primers prior to incubation at 37 ˚C for 1 hour.[14] The primer sequences used are shown in table 1.

The first PCR amplification round was performed in a total volume of 50 µl containing both outer forward and reverse primers, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 2 U Taq polymerase (Finnzymes, CA) and 10 mM dNTP. The PCR conditions comprised one initial denaturation cycle at 94^0C for 1 min, followed

by 35 cycles at 94^0C for 1 min (denaturation), $54\ ^0$C for 1 min (primer annealing), 72 ^{0}C for 1 min (extension) and a final extension step at $72\ ^0$C for 7 min. The first round PCR product was further amplified using the inner forward and reverse primers. Apart from that, the conditions were identical to those applied in the first round. The second round PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide. Under ultraviolet light, the PCR products became visible. Beta actin served as an internal control. The primers used are shown in table I.

Molecular characterization:

To confirm the PCR products' specificity and further characterize them, the second round PCR products were purified from the agarose gel with the Perfectprep Gel Cleanup Kit (Eppendorf, Westbury, NY) according to the manufacturer's specifications and subsequently subjected to 2 % agarose gel electrophoresis in order to ascertain their purity.

To determine the concentration of the amplified DNA, we measured every sample's absorption at 260 nm in a UV spectrophotometer (Shimadzu UV 160 A, Tokyo, Japan). The concentration was calculated according to the formula 1 OD 260 = 50 μg double-stranded DNA. Between 10 and 30 ng/μl (3-6 μl) of each DNA sample were subjected to cycle sequencing using 4 μl of dye terminator, 2 μl of DNA sequencing buffer (Big Dye Terminator V.3.1 Cycle Sequencing Ready Reaction, Foster city, CA) and 3.2 pmol of specific primer in a final reaction volume of 20 μl in a thermocycler (9600 Perkin Elmer Cetus, Norwalk, CT). This amplification round was performed according to the manufacturer's specifications, using both inner forward and reverse primers to amplify the particular DNA strand of interest for further sequencing (Theamboonlers et al., 1999). The extension products were subsequently purified from excess unincorporated dye terminators by ethanol

precipitation according to the manufacturer's specifications (ABI Sequencing kit, ABI, Foster city, CA) and subjected to sequence analysis by the ABI Prism 310 Genetic Analyser.(ABI, Foster city, CA).

For all subsequent steps, we referred to the ABI Prism 310 Genetic Analyser user's manual. The sequences thus obtained were analyzed by Navigator program and submitted to the GenBank database (accession numbers AY 158463-5 and AY550148-56 for N gene; AY550157-66 for F gene; AY550167-75 for L gene). Nucleotide sequences were also compared with sequences previously published in GenBank applying the BLAST program (www.ncbi.nlm.nih.gov/Blast)

Phylogenetic analysis

DNA sequences were aligned by CLUSTAL X software (EMBL, Heidelberg, Germany). Phylogenetic trees were constructed by the PHYLIP 95 program, using DNADIST followed by the neighbor-joining method supplied by the Tree **V**iew 32 program.

Human respiratory syncytial virus detection.

We also screened for hRSV RNA to both detect hRSV infection and perform subtyping. The details pertaining to this method have been published elsewhere.[17]

Statistical Analysis

The data were expressed as mean ± SD where applicable. Analysis was performed by the Mann-Whitney U test. (SPSS for Windows, version 11.) All p values <0.05 were considered significant.

Results

Of the 220 specimens tested, 134 had been obtained from male and 86 from female subjects, respectively. Their mean age was 13.9 ± 11.9 months. One hundred and eighteen were positive for hRSV.(54%) Twelve were positive for hMPV(5%). There were two infants co-infected with hRSV subtype B. Eleven positive specimens (91%) originated from hospitalized infants and children. All had presented with fever and cough (100%), ten (83%) had upper respiratory tract symptoms at the time of initial examination. Their mean age was 22.3 ± 11.5 months, significantly higher than that of infants with RSV infection (11.2 ± 9.8 month, p=0.001). We noticed a 3:1 ratio of infected boys versus girls (9:3) thus confirming previous knowledge of males being associated with an increased risk of having lower respiratory tract disease, as well as other respiratory virus infections.Twelve hMPV isolates obtained from patients suffering from lower respiratory tract illness were used for the current study.

Phylogenetic analysis

Previous studies have shown two distinct phylogenetic lineages of hMPV.[18,19] Our sequences were aligned by CLUSTAL X software package. Genetic distances were calculated employing the Kimura 2-parameter method and phylogenetic trees were constructed by the neighbor-joining method of the PHYLIP 95 program. The phylogenetic relationships determined for N gene of hMPV, of our study including previously published sequences as follows: AY158463-65 and AY550148-56 from Thailand; AY145272-85 from Canadaa; AY355319, AY355325-28 and AY355330-35 from Nethelands; AY297748-49 from USA and AY17659 from APV-C. For F gene are: AY550157-66; Thailand, AY152847 and AY15285; Spain, AY145287-92, AY145294-97 and AY145299-301; Canada, AY297748-49; USA,

AB18701, AB119484, AB119486-87, AB119489-94, AB126605-07, AB126611-13 and AB113371; Japan and AY187152; APV-C. For L gene we used: AY550167-75; Thailand, AY297748-49; USA, AY168973-84 and AY168986-87; Italy, AY16949, AY216951-52, AY16954-55, AY216958, AY216961, AY216967, AY216969, AY216971, AY216974 and AY216983-84; UK and U65312; APV-A.

Phylogenetic analysis based on the N and F genes showed two groups of hMPV sequences. The sequence similarities within each group exceeded 97% and those between groups were 87-88% and 81-84%, respectively. In addition, phylogenetic analysis based on the L gene of our hMPV sequences and the sequences previously reported showed at least two groups of hMPV sequences. The sequence similarities within each group exceeded 98%. Similarly, the sequence similarities between groups were 95-96%. The phylogenetic tree analysis comparing between different geographical areas **is** shown in Figs.1-3

The clinical diagnosis given to the twelve patients with human metapneumovirus infection of the lower respiratory tract was acute bronchiolitis in 5 (42%), viral pneumonia in 6(50%) and exacerbation of BPD triggered by viral respiratory tract infection in 1 OPD case (8%). The mean duration of hospitalization required was 6.8 ± 3.6 days, significantly exceeding that experienced by infants infected with hRSV (3.5 ± 1.2; $p<0.05$). Eleven hospitalized children underwent chest radiography, which without exception conveyed abnormal results in that the radiographs of most of these children showed bilateral perihilar infiltrates.(6, 54%) Their WBC ranged between 6330 and 16450/mm^3 (median, 10500). A summary of these findings is shown in tables 2 and 3. The majority of patients (75%) were treated with antibiotics. One patient underwent an infant lung function test, which showed small airway obstruction devoid of response to a bronchodilator.

Discussion

Here we describe the characterization of a novel human respiratory metapneumovirus, isolated from 12 Thai infants and children with lower respiratory tract infections (11 admitted, 1 OPD). The children presented mostly with clinical symptoms similar to hRSV infection, ranging from upper respiratory tract disease to acute bronchiolitis and pneumonia, which has also been stated by a recent report.[20] As is the case with hRSV-infected children, young infants or those with underlying diseases (complex congenital heart disease, 33%) appeared to require extended hospitalization. Moreover, young children with hMPV infection and especially those infants with underlying diseases required significantly longer hospitalization than patients with hRSV infection. This may be attributed to the fact that the majority of young children infected with hRSV had no underlying disease.[17] In contrast to the observations made by Williams JV *et al* [21] according to which hMPV infection mostly occurred in the first year of life, we found it to be more common in the second year. This may suggest differences in host susceptibility or molecular characteristics of this virus. Our data also indicated that hMPV infection could potentially become life-threatening especially in immuno-compromised or certain high risk individuals.[22-24] In addition, it demonstrated that hMPV could be an important cause of lower respiratory tract infection in infants and young children. We found hMPV infection in twelve patients (5%) compared to 7.5% reported from The Netherlands [25], 5.5% from Hong Kong [26], 3.9% from the CDC, USA [27] and 6.6% from a French study.[1] Furthermore, our data have shown that the primary manifestation of hMPV infection was pneumonia (50%) followed by acute bronchiolitis.(42%) Therefore, there is a strong association between hMPV and lower respiratory tract infections.

The role of viral respiratory tract infections in acute and chronic asthma has been the subject of enhanced research interest. One of our children infected with hMPV displayed acute exacerbation of BPD suggesting that, as in hRSV or rhinovirus infections, hMPV infection could possibly induce exacerbation among children with reactive airway disease [26,28-29] although other studies have found asthma to be more frequently associated with rhinovirus than with hMPV.[30] In addition, similar to other viruses, hMPV is subject to a seasonal pattern; hence, co-infection with other respiratory viruses might be possible, but the role that hMPV plays as a co-pathogen has to date remained unclear.[2,5,26] In our study, we found two infants co-infected with hRSV, which may contribute to illness severity. According to a recent study reported from Europe, 70% of infants hospitalized with RSV infection and requiring mechanical ventilation were also co-infected with hMPV.[12] As we screened only for two viruses (RSV & hMPV) the extent and significance of co-infection with other respiratory pathogens will require further studies.

Future active hMPV surveillance will be required to determine a more comprehensive spectrum of diseases potentially caused by this pathogen. Moreover, asymptomatic infants and children were not tested and consequently, the overall prevalence of hMPV infection could not be ascertained. Furthermore, we limited our study to children below the age of five and thus, both clinical features and incidence rate among older individuals remain to be determined. Although the identification of viral nucleic acid in respiratory secretions does not unequivocally prove that hMPV was responsible for the patients' symptoms, the association between respiratory tract illness and presence of the virus suggests a causative role. Previous studies have shown two distinct major hMPV phylogenetic lineages based on analysis of the limited sequences available. [15] Concurring, our phylogenetic analysis identified 2

genetic lineages of hMPV, which co-circulated during the same year. Both N and F gene lineages displayed in excess of 80% similarity between each group and more than 95% was found in the L gene.

In summary, hMPV presents new opportunities and challenges to our efforts to better understand human respiratory tract diseases. Advances in molecular analysis will assist us to identify the differences in genetic lineage of this important virus. At present, no diagnostic kit has yet become commercially available. Therefore, diagnostic tools exhibiting enhanced practicality and sensitivity, yet less costly ought to be developed so that a viable estimate of the extent of illness caused by hMPV among the public can be obtained.

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Area of research interest : Basic science and clinical trials related to viral respiratory tract infection; especially RSV or newly emerging respiratory virus, High frequency oscillator ventilation, Sepsis/septic shock, Inhaled nitric oxide in ARDS.

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Figure1: Phylogenetic analysis of N gene of human metapneumovirus (hMPV) compared to other sequences among different countries applying the Phylip 95 program. Vertical branches are labeled with the accession number in the GenBank database and countries (AY158463-65 and AY550148-56; Thailand, AY145272-85; Canada, AY355319, AY355325-28 and AY355330-35; Netherlands, AY297748-49; USA and AY17659; APV-C).

Figure 2: Phylogenetic analysis of F gene of human metapneumovirus (hMPV) compared to other sequences among different countries applying the Phylip 95 program. Vertical branches are labeled with the accession number in the GenBank database and countries (AY550157-66; Thailand, AY152847 and AY15285; Spain, AY145287-92, AY145294-97 and AY145299-301; Canada, AY297748-49; USA, AB18701, AB119484, AB119486-87, AB119489-94, AB126605-07, AB126611-13 and AB113371; Japan, and AY187152; APV-C).

Figure 3: Phylogenetic analysis of L gene of human metapneumovirus (hMPV) compared to other sequences among different countries applying the Phylip 95 program. Vertical branches are labeled with the accession number in the GenBank database and countries (AY550167-75; Thailand, AY297748-49; USA, AY168973-84 and AY168986-87; Italy, AY16949, AY216951-52, AY16954-55, AY216958, AY216961, AY216967, AY216969, AY216971, AY216974 and AY216983-84; UK, and U65312; APV-A).
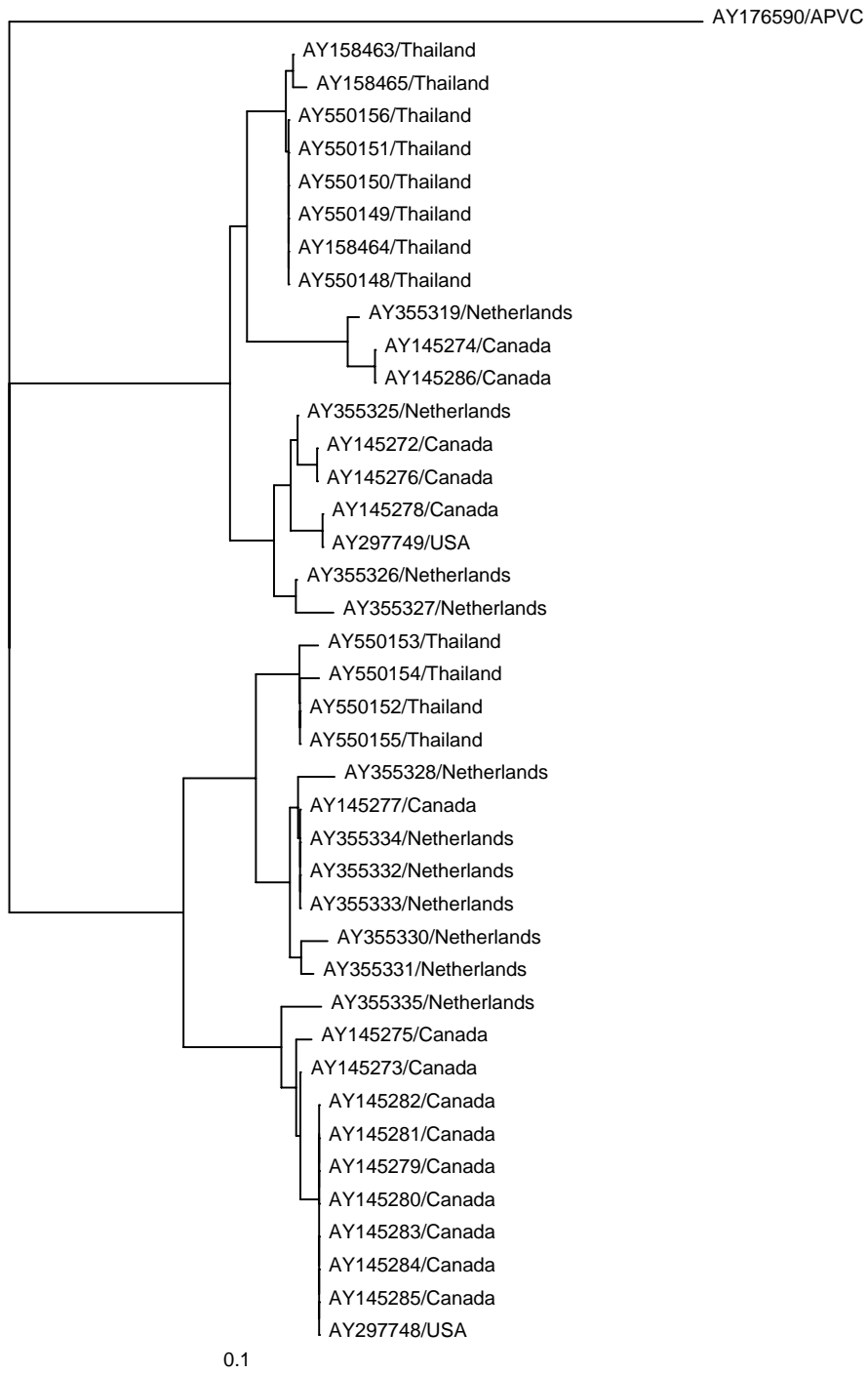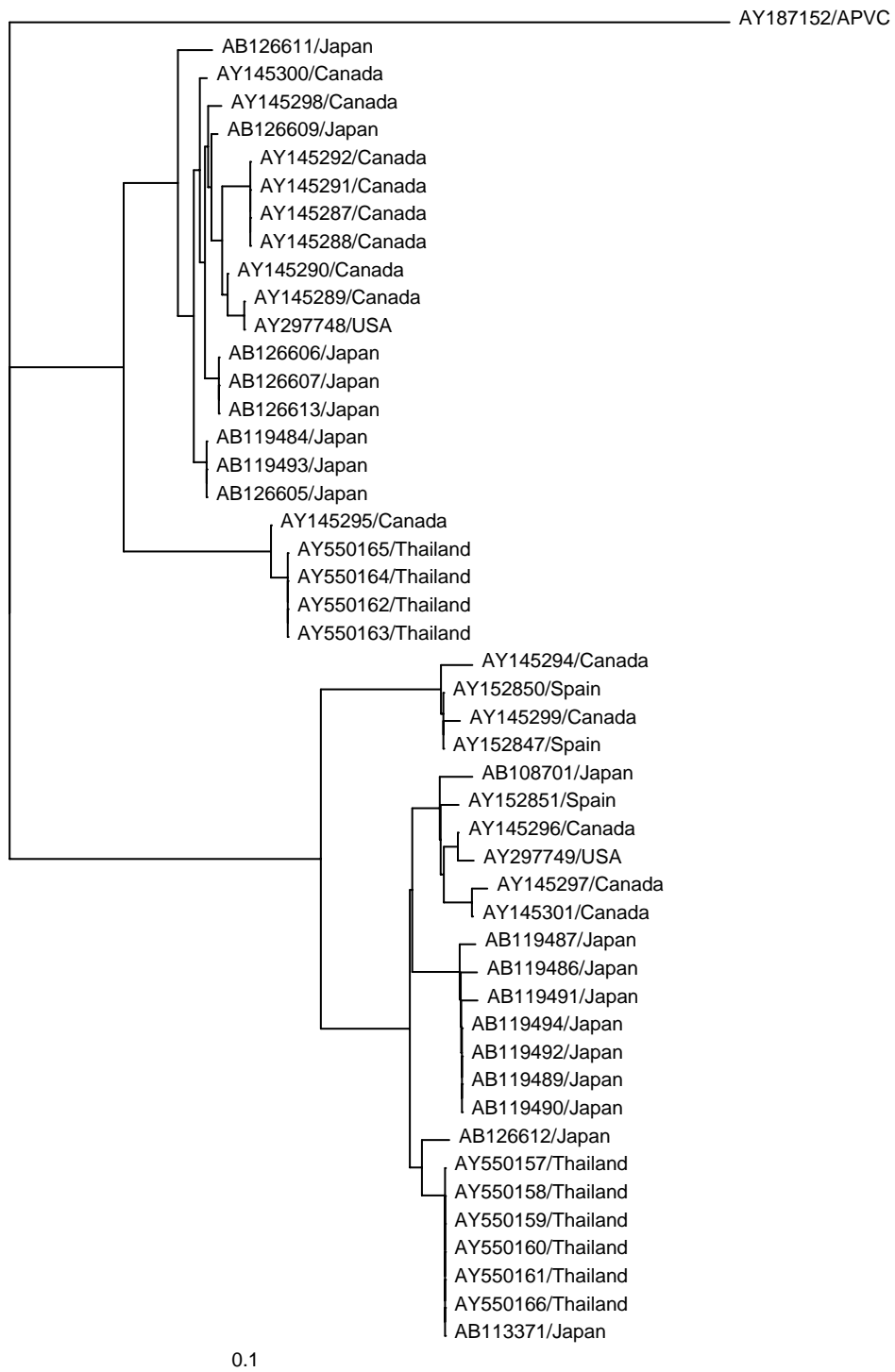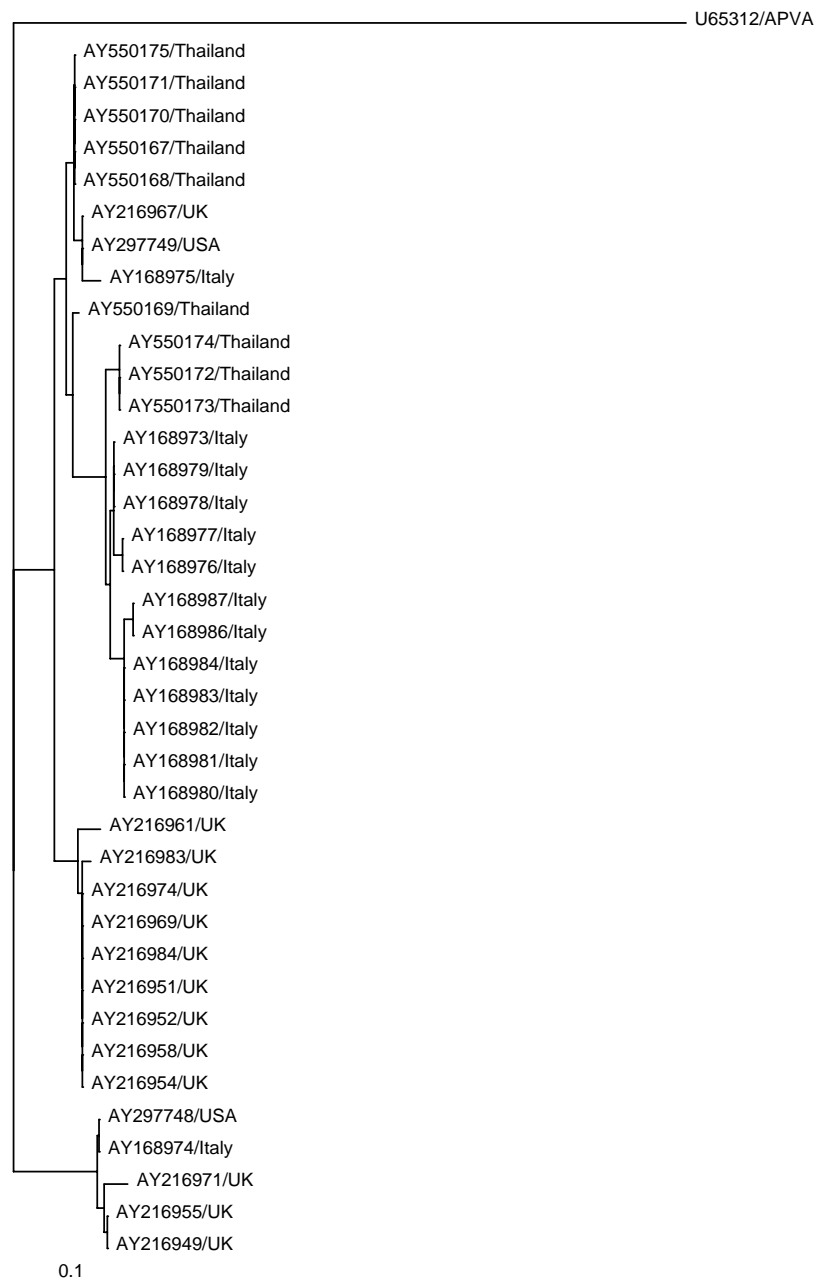
Figure1:

Figure 2**:**

Figure 3:

Table. I：N, F, L gene and beta –actin primers used in this study.

Gene	Primers	Sequences	Position	product size	Reference
N	Outer forward primer (MPVP F)	5′ ACGGGGTAGAGAAGAGCTGG 3′	389-408		-
	Outer reverse primer (MPVP R)	5′GCAAAGTTGGGACAGTTGGC 3′	1004-985	259 bp	-
	Inner forward primer (MPVN F)	5′ GCATCAACCATAGAAGTGGGAC 3′	556-577		-
	Inner reverse primer (MPVN R)	5′ GCATTGTTTGACCGGCCCCA 3′	814-795		-
F	Outer forward primer (MPVS F)	5′ GGTTCCTAAATGTTGTGCGG 3′	3647 - 3666		-
	Inner forward primer (MPVF1f)	5′ CTTTGGACTTAATGACAGATG 3′	3704-3724	450 bp	[29]
	Reverse primer (MPVF1r)	5′ GTCTTCCTGTGCTAACTTTG 3′	4153-4134		[29]
L	Outer forward primer (MPVS L)	5′ GCACTAAGTGAGAGATTTGGG 3′	11052-11072		-
	Inner forward primer (BF 44)	5′ CATGCCCACTATAAAAGGTCAG 3′	11336-11357	171 bp	[8]
	Reverse primer (BF 2)	5′ CACCCCAGTCTTTCTTGAAA 3′	11506-11486		[8]
β-actin	Forward primer (β-actin F)	5′ ATGCCATCCTGCGTCTGGACCTGGC 3′	591-615	606 bp	[30]
	Reverse primer (β-actin R)	5′ AGCATTTGCGGTGCACGATGGAGGG 3′	1196-1162		[30]

Table II : Demonstrate the clinical manifestations of hMPV infection.

Pt No(G)	Age months	Hospitalization (days)	Diagnosis	Underlying disease	Signs/Symptoms	Chest Radiograph Findings
1.(M)	9	6	Ac. bronchiolitis	No	Fever, rhinorrhea Profuse diarrhea, wheezing	Interstitial with LL infiltrates
2.(F)	38	3	Viral pneumonia	No	Fever, severe cough, fine crepitations both lungs	Perihilar infiltrates Patchy RUL/RML inf
3* (F)	14	11	Viral pneumonia	Pulmonary a sling Tracheal stenosis	Fever, productive cough tachypnea, coarse rales, chest retraction	Perihilar
4.(M)	36	9	Viral pneumonia	Truncus arteriosus	Fever, protract cough Bil medium rales	Inf Hyperaeration
5.(M)	12	3	Viral pneumonia	No	Fever, clear rhinorrhea Occasional rhonchi bil	Perihilar inf N/D
6(M)	36	-	URI/RAD	BPD	Fever, rhinorrhea, cough Exp wheezing	Perihilar
7(M)	36	2	Viral pneumonia	No	Fever, productive cough Fine crepitation, rhonchi	Infiltrates Rt middle
8.(M)	24	14	Acute bronchiolitis Viral pneumonia	Complex Congenital heart	Fever, cough, tachypnea, Diffuse wheezing & Rhonchi	Lobe infiltrates Bil perihilar
9(M)	18	6	Acute bronchiolitis	No	Fever, productive cough, bil fine crepitations	Infiltrates Small
10*(M)	9	7	Acute bronchiolitis	Complex Congenital heart	Fever, clear rhinorrhea Tachypnea, diffuse rales	Patchy RML Perihilar
11(F)	24	8	Acute bronchiolitis	No	Fever, dry cough LLL rales,exp wheezing	Infiltrates Hyperinflation Perihilar, RLL
12(M)	12	6		Congenital Rubella syndrome	Fever, intractable cough Tachypnea, wheezing	infiltrates

* Co-infection with RSV subtype B; n/d = not done

URI**:** upper respiratory tract infection**;** LLL**:** left lower lobe**;** RLL**:** right lower lobe

RML**:** right middle lobe**;** * co-infection with RSV subtype B

N/D**:** not done

Table III: Demonstrate the clinical presentations of hMPV compared to hRSV.

Characteristics Total no 220	hMPV	hRSV	p value
No. Positive	12 (5.4%)	118 (53%)	
Age (months) (Mean±SD)	22.3 ± 11.5	11.2 ± 9.8	p=0.001
Sex (M:F)	9:3	68:50	NS
Fever (˚C) (Mean±SD)	37.7 ± 0.5	37.9 ± 0.74	NS
Acute Bronchiolitis	5 (42%)	64 (53%)	
Pneumonia	6 (50%)	54 (47%)	
Hospitalization days	6.8±3.6	3.5±1.2	p<0.05