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Epidemiology Study of Human Metapneumovirus in Malaysia among Paediatric Children below 4 years of age, 2012

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Abstract

We have done a specific study on the molecular epidemiology of Human Metapneumovirus cases which was found in Malaysia among paediatric children below 48 months of age from January till December 2012. These paediatric cases were isolated from the rest of those entire positive below 48 months of age. The total number of the positive cases below 48 months of age is 53 cases out of the general population of 438 positive cases. We performed phylogenetic analysis on these patients and a seasonal prevalence hMPV incidence was observed in the month of November. A high level of sequence identity was observed in the A2 subgroup and no amino acid substitution was found compared to the strains observed in Malaysia and other countries. The pairwise distance among the strains belonging to the predominant subgroup A2was0.0925 suggesting highly homologous with seasonal epidemics.

Keyword: Molecular epidemiology, human metapneumovirus, polymerase chain reaction (PCR), paediatric, phylogenetic analysis.

Introduction

Malaysia, a tropical country, is located in the Southeast Asia region. This country separated by the South China Sea into two similarly sized regions, Peninsular Malaysia and Malaysian Borneo.The country is also surrounded with Thailand in the northern region, Singapore in the southern region, of Peninsula Malaysia, Indonesia in the Southeastern of Borneo and Brunei neighbouring the Northeast of the Sarawak state of Borneo. There 3 main seasonal variation of rainfall with high rain fall in the east coast states from November till January; from October till November and April till May in the north western region; and from May to August in the southwest coastal area of the peninsular states.

Respiratory viral infection mostly occurs during the winter season and in the temperate climate[1]. The viral community outbreaks during these seasonsare often associated with the increase of nosocomial transmission, risking the immunocompromised patients and children withcardiac and pulmonary disease which could lead to severe complications[2].

Human Metapneumovirus (hMPV) has been identified as a new causative agent for upper and lower viral respiratory tract infection in children and adults worldwide. It has been identified in patients whom screening tests for other viral pathogens had been[3]. The virus is ranked as the second leading cause of death worldwide in children >5 years of age[4]and reinfection of this virus occurs throughout life[5].

Since the epidemiology of hMPV distribution based on the age <4, sex, race andmonsoon period has not been determined, hence we analysed the epidemiology of hMPV based on these criteria in the country.

Material and Methods

About 1580 respiratory samples obtained from hospitals around the country were received by the Virology Unit, Institute for Medical Research from January 2012 till December 2012. Samples in the form of throat swap (T/S), nasopharyngeal aspirate (NA) and nasal swap (NS) were received for the detection of upper and lower respiratory tract infection. From this large sample size,438 patients samples were from paediatric<4 years were screened for hMPV.

From this, 53 patient samples were found to be positive for hMPV infection. All of these patients had clinical history of $\geq 38^0$C fever, runny nose,cough and wheezing, and were further grouped from 0-12 months, 12-24 month 24-36 month and 36-48 months of age, with the mean ± standard deviation [SD] of 13.25 ± 17.6.All of these samples were received in viral transport media (VTM) and kept refrigerated at 4^oC until it is processed for viral culture and immunofluoresence assay. After samples had been processed, it is cultured into a culture tube for

viral growth identification and for immunofluoresence assay. The remaining of the culture fluid is stored in -70^{o}C until it is tested for PCR.

Carcinoma Human type II Alveolar Epithelial (A549) cell lines were used to culture the virus. This cell lines were obtained from ATCC (CCL-185). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with10% of foetal calf serum used to grow the cells while 2% of foetal calf serum was used to maintain the cell lines. Once the cells are 80-90% confluent, the cell lines were infected with the patient samples and incubated in 5% CO_2 incubator, and were observed for CPE up to 14 days of incubation.

After the virus has been cultured into the A549 cell line, detection of the hMPV viral antigens was done using the D^3 DFA Metapneumovirus Identification kit, according to the manual (Diagnostic Hybrids, USA).This commercial kit detectsthe viral antigens by immunofluorescence assay using a blend of three monoclonal antibodies (MAbs), from patients with signs and symptoms of acute respiratory infection.

Culture fluid frompatients was placed on a glass slide and was allowed to air dry before it could be fixed with acetone. Then, DFA Reagent was added to the cells and was incubated for 15 to 30 minutes at 37^{o}C in a humidified incubator. After the incubation step, the stained cells are washed with Phosphate Buffered Saline (1X PBS).

A drop of Mounting Fluid which was supplied was added and a coverslip was placed carefully on the prepared cells. Finally, the cells are the examined under a fluorescence microscope**.** Infected cells will appear apple-green, compared to the uninfected which was stained red by the Evans Blue counter-stain. Samples were then proceeded to PCR to detect the hMPV sublineage.

About 150µl of Viral RNA was extracted using theQiagenQIAamp Viral Mini extraction kit and was eluted with AVE Buffer. After the RNA extraction, the pureviral RNA was amplified using the One-Step RT-PCR kit from Qiagen, which contained 5µl of 5X OneStep RT-PCR Buffer, 400µM dNTP, 0.6µM of each primer[MPVF1 For: (5'-CTTTGGACTTAATGACAGATG-3') and hMPVF2 Rev: (5'-GTCTTCCTGTGCTAACTTTG- 3')], which corresponds from the nucleotide3704– 4153 [6].

These primers amplified a 450 bp fragment and the amplicons were purified with a PCR purification kit (Qiagen, Germany) before it could be sequenced.

The PCR products obtained were confirmed positive by commercial automated sequencing followed by computer analysis using BLAST software from the National Centre for Biotechnology Information (NCBI).

Results and Discussion

A Phylogenetic tree based on the sequenced analysis was aligned with the sequences obtained from the GeneBank. The multiple sequence alignment was done using CustalW alignment program of Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.03 [7]. The average pairwise Jukes–Cantor distance[8] was found to be 0.0899 indicating that the data was suitable to construct Neighbour-Joining trees[9]. Trees were constructed using the p-distance nucleotide substitution model, with 1000 bootstrap replicates, using the Mega 5.03 software. The pairwise distance among the strains belonging to the predominant subgroup, hMPV A2 was 0.0925 suggesting highly homologous with seasonal epidemic with the average number of base substitutions per site from the average sequence pairs was 0.0986 with the standard error estimated 0.0111. Basedon the phylogenetic tree, the data shows well separated groups of all 4 subtypes with 9 (17%) patients having infection with hMPV A1, 26 (49%) patients with hMPV A2, 7 (13%) patients with hMPV B1 and 11 (21%) patients with hMPV B2. Out of the 53 patient samples<4 years of age tested, hMPV A2 was the highest number which contributes to 26 (49%) whereas the overall total of the other subtypes, hMPVA1, hMPVB1 and hMPVB2 is 27 (51%). This reveals that hMPV A2 prevalence occurred. The occurance of hMPV A2 positive against the overall paediatric population was 5.9%. Based on the phylogenetic tree, the similarity of the sequence was accordance with the topology of the tree. This phylogenetic analysis was constructed based on the F gene of hMPV. The nucleotide identity between subgroups A and B was 87 %–89 % with the nucleotide identity between subgroup A1–A2 and B1–B2, of 96%–97% and 98.2%–99.7% between the both subgroups A1–A2 and B1–B2, respectively. Based on the phylogenetic tree, it can be concluded that the subgroup A2 was the most divergent. The similarity of the F gene sequence shared a (92.8%–99.6%) nucleotide identity figure 1.

The figure 2 shows the number of positive cases respiratory viruses detected in Malaysia in the year 2012. The point denotes the positive cases of hMPV detected in children <4years of age. From the chart we could see that in the month of Jan (10 cases) Sept (6 cases), Oct (5 cases), Nov (11 cases) and Dec (15 cases) high number of positive caseswere observed. Thiscould be due to the climate change, as Malaysia is a tropical rain forest country and this time of the year is the Monson period. The hypothesis is that, this Monson period flu and rhinitis is very common. When a patient is down with a common cold, the chances of this virus attacking the patient are high. Hence, the virus can be associated with low immune system. When someone's immune system is low, this virus's potency will be high to infect the individual. To support this theory, *Tameriuset al.,* quoted that Flu epidemics strike during the winter in temperate regions, but the seasonality of flu is less clear in the tropics. The author also indicated that places where outbreaks tend to occur during rainy seasons or year-round suggests that rain and humidity is key factors in tropical regions[10].

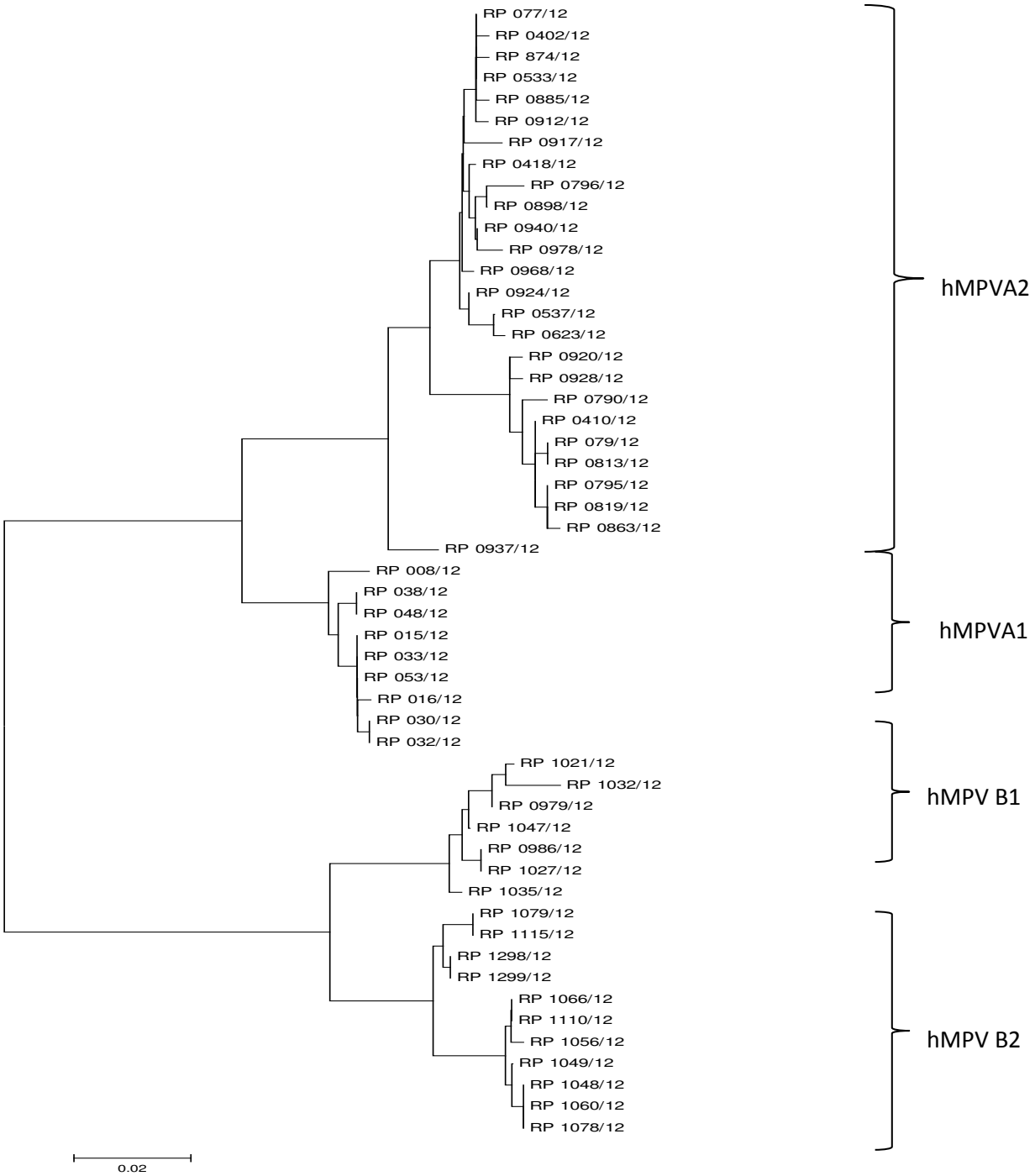

Figure-1
Phylogenetic tree of hMPV based on the F gene sequences. The phylogenetic tree was constructed after neighbour-joining analysis based on the nucleotide sequences of the 450bp fragment of the hMPVfusion (F) region corresponds from the nucleotide3704– 4153 with 1000 bootstrap replicates

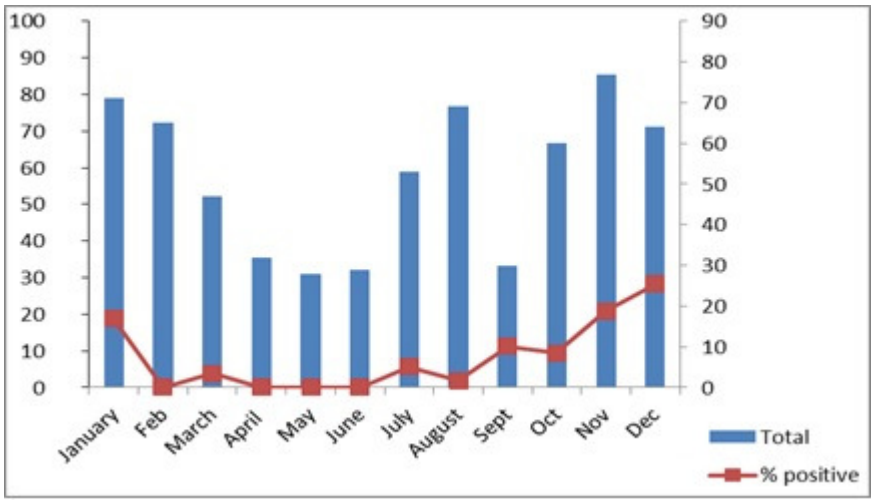

Figure-2
Monthly distribution of hMPV detected in Malaysiavs, Number of sample in the year 2012

Table-1
Number of positive cases based on the age group

Age in Months	No of +ve Cases	%	Remark
0-12	39	73.6 %	The higest incident
12-24	10	18.7%	Moderate Incident
24-36	3	5.7%	Moderate Incident
36-48	1	1.9 %	The Lowest Incident
Total	53	100%	Total

Out of the total number of general population of positive which were 625 casesamong patients with respiratory infection, only 53 were found to be positive for hMPV in paediatric patients <4 years of age. In this study, we have only taken these 53 cases and did an intensive study keeping 625 as the general positive cases.

We further did grouping of this positive cases in to more complex clusters which has only two sub division, in order to narrow down our study. The below are the statistics.

Table-2
Number of positive cases based on the cluster group

Age in Months	No. of +ve Cases	%	Remarks
0-24	49	92.5%	The higest Incident
24-48	4	7.5%	The lowest incident
Total	53	100%	Total

We found that children in the age group 0-12 months were the highest with 73.6% were by 39 positive cases recorded. The 12-24 months and 24-36 months fall under the moderate incident group. 18.7% and 5.7% respectively. The lowest positive hMPV cases we recorded in 36-48 months with just 1.9 % which is only 1 case. This shows that the high number of positive cases was observed in the 0-12 months of age group. This factor proves that new born children has the lowest body resistance compared to the other age groups which further provides evidence that this virus has the highest potency level in patients whose immune system which is weak. As the age increases, the level of immune system of the paediatric child increases and we recorded lower occurrence of the incident. The above fact is further supported through our more complex cluster system where we would only have 2 age groups, which are the, 0-24 and 24-48 months of age.In this we found that the 0-24 has the highest positive cases which are 92.5% compared to 24-48 which has 7.5 %.Hence we conclude that, age and immune system is a key factor which plays an important role in the occurrence of this hMPV viral infection. In a study done by Researchers from the Albert Einstein College of Medicine of Yeshiva University, the mechanism by which aging may compromise the ability of the immune system to fight infections was uncovered. The study by Cannizzo., et al., 2012 quoted thataging can worsen the body's overall ability to mount an effective immune response[11].

Gender: The overall rates oh respiratory infection was reported in the male children with 357 cases (57%) compared to female children which was 268 cases (43%). A high number of Paediatric patients with hMPV infection was also detected in the male 32(60%) compared to the female paediatric 21(40%). These finding is accordance to a study done by a group of researchers from Denmark. From their study, they came up with a hypothesis that gender plays a role in the susceptibility for respiratory infections in early childhood. The researchers also

came up with a conclusion saying that the overall morbidity and mortality rates in childhood are higher in males than females[12].

Conclusion

In conclusion, we found that the prevalence of hMPV is found in Malaysia. With 53 positive cases under the age of 4 years this number could be considered for a high mortality in paediatric cases, where age, climate and sex is the major factor effecting children below the age of 4.In order to determine the incidence of this virus in the Malaysian society, more research should be done to determine the molecular epidemiology of this viral disease in this country. The practice would be important for the development for future therapeutic and preventive strategies to fight respiratory viral infection in children and adults.

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