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Incidence of Human Metapneumovirus in Hospitalized Patients Admitted for Respiratory Illness in Malaysia

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Abstract

Human metapneumovirus (hMPV) is an emerging human respiratory pathogen. It clinically resembles respiratory syncytial virus, which causes both upper and lower respiratory tract disease, and has been associated with serious respiratory illness particularly among infants and young children. To date, no published data on the incidence of hMPV infection has been reported in Malaysia. This study was conducted from February 2010 till March 2012. In this period of study, the Institute for Medical Research (IMR), Kuala Lumpur received a total of 2600 respiratory samples from hospitalized patients in Malaysia, suspected with upper and lower respiratory tract infection. Out of this number, 625 patient samples were negative for RSV, Adenovirus, Parainfluenza 1, 2, 3, Flu A and Flu B, which are the routine respiratory virus detection done in the laboratory of the department. The negative samples were then analyzed for hMPV by viral culture, and Direct Immunofluorescence assay, for the detection on hMPV in samples. Further confirmation by doing reverse-transcription PCR was done on all 625 respiratory samples. This method targeted hMPV polymerase (L) gene of 170bp fragment. A phylogenetic tree based on 450 bp fragment of the F gene was also constructed. The results of F gene region showed (93%) homology to the reference sequence from the NCBI AAQ67695.1. Out of all the samples analyzed, 130 respiratory samples (20.8%) were positive by viral culture method and by Immunofluorescence assay and 167 (26.7%) were positive for Human metapneumovirus by PCR. The most common clinical findings include fever, cough, and wheezing. Most of the hMPV positive cases were detected were from hMPV A2 sublineage.

Keyword: Respiratory, human metapneumovirs, viral culture, direct immunofluorescence assay, RT-PCR, phylogenetic analysis.

Introduction

In the year 2000, out of 10 million deaths among children less than 5 years of age throughout the world, 1.9 million children died from acute lower respiratory tract infection[1]. Respiratory tract infections is said to be the leading cause of morbidity and mortality worldwide. The most common illness regardless of age or gender is said to be, acute respiratory tract infection (ARI). For children <5 years old, respiratory tract infections are ranked as the second leading cause of death[2].

In the US, the incidence of lower respiratory tract infection is high and causes about 19% to 27% of hospitalizations in children under the age of 5 years[3,4]. It is the eight leading cause of deaths, with approximately, accounting for 56,000 deaths annually. Even though the mortality rate in children decreased by 97 % in the past century, respiratory diseases continues to be a leading cause of pediatric hospitalization and outpatient visits in the country[5].

In a study conducted in University Malaya Medical Centre, Kuala Lumpur by Khor, et al., 2012, respiratory illness was a significant cause of morbidity in hospitalized children in Kuala Lumpur. According to the study conducted between 1982 and 2008, out of 10269 samples obtained from children ≤ 5 years 26.4% were positive for the common respiratory viruses[6].

Human Metapneumovirus (hMPV) was first discovered and reported as etiological agent of acute respiratory illness (ARI) in 2001. It is a common respiratory pathogen, particularly in infants and young children. However, based on the previous studies conducted, hMPV is responsible from 5 to 10% of hospitalization of respiratory cases in neonates and children[7,8]. The virus is classified under the Pneumovirinae subfamily of the Paramyxoviridae family and, is a negative single-stranded RNA-enveloped virus with 13.3kb in size and encodes nine proteins, similar to the Human Respiratory Syncytial virus, except for the lacking of the non-structural proteins[9]. The morphology of the virus is similar in appearance as other Paramyxoviridae family members. The viral particles are said to be spherical, filamentous or even pleomorphic in shape with measuring from 150-600 nm in diameter. The particle of this virus contains 15 -nm projections from the surface. According to the sequence analysis of hMPV isolates, two main lineages

have been identified, hMPV A and hMPV B and each group is further subdivided into 2 more lineages: A1, A2, and B1, B2. In 2006, a novel sub-cluster within the viral subgroup A2 was identified, which was named A2a and A2b[10]. Based on a study done by Biovin et al., the predominant strain which was circulating was from hMPV A[11]. In Germany however, the new sub- cluster A2b, was found to have one-third of the cases in two of the winter season[10]. Both virus genenotypes have been reported circulating in various countries in the Americas, Europe, and Asia[12].

During the initial identification of this respiratory virus, the replication of this virus in tertiary monkey kidney (tMK) cell, was slow and the replication rate in cells like the Vero cells and the human adenocarcinoma (A549) cells was said to be poor. Furthermore, hMPV was not successful being propagated in Madin Darby canine kidney (MDCK) cells and in the chicken embryo fibroblasts cells (CEF). The cytopathic effect (CPE) of this virus presents a syncytia formation followed by rapid internal disruption of the cell growth which leads to detachment from the culture plate and this scenario usually takes place between the 10 to 14 days of post inoculation[13].

Human metapneumovirus was first detected by extracting RNA from tMK infected cells using reverse transcription method using primers specific to known paramyxoviruses[14,8]. This assay is more sensitive and reliable than culture techniques and takes lesser time to detect the presence of the viral pathogen. Since then, this molecular method has become the most preferable mode of detection and diagnosing of hMPV in patients with respiratory infection. The most common gene of target for PCR is the nucleoprotein (N) and polymerase (L) genes. This is because of the conserved nature of all the hMPV strains[14,15]. This negative-strand RNA genomes contains open reading frames (ORFs) which encodes three putative viral envelope glycoproteins, which is the F (fusion) protein, G (attachment) protein, and SH (short hydrophobic) proteins (16). From these three genes, the F gene has the most highly conserved gene with 33% identity on the amino acid level between hMPV and RSV. Similarity among several features exists in the F protein in hMPV when compared with this protein in other paramyxoviruses. These includes the distribution of cysteine residues, a putative cleavage site (a distinguishing feature of a fusion proteins), fusion domains, and anchor sequences[16].

The virus is genetically closely related to avian metapneumovirus (formerly known as turkey *rhinotracheitis* virus). These two viruses are classified under the genus Metapneumovirus, with hMPV the first in this genus to cause disease in humans. Although it is hypothesized that the human virus originated from birds, the serological evidence shows that hMPV has been widespread in humans since at least 1958. This suggests that, zoonotic divergence before this time existed[13].

After the first detection of hMPV in 2001 by researchers from Netherlands, the incidence of the virus has been reported in many European countries, Asia and North America. However, there are no published data available on the incidence of Human Metapneumovirus in Malaysia.

The incidences of hMPV infection have not been described in Malaysia. The main aim of this study was to determine frequency of incidence of this hMPV viral by utilizing a sensitive and reliable method and also to identify the epidemiology and clinical characteristics of hMPV among hospitalized patients in Malaysia.

Material and Methods

Clinical samples: The study was conducted in the Virology Unit, Institute for Medical Research, Kuala Lumpur, from February 2010 till March 2012. A total 2600 patients samples which includes, throat swap (T/S), nasopharyngeal aspirate (NA) and nasal swap (NS) were received for the detection of various respiratory tract infection. Out of this, 625 respiratory patient samples were negative for other respiratory viruses including, respiratory syncytial virus (RSV), Adenovirus, Parainfluenza 1, 2 and 3 and Influenza A and B viruses. The patients that were negative for these viruses had a clinical history of $\geq 38^0$C fever, runny nose, cough and wheezing. All of these samples were received in viral transport media (VTM) and kept refrigerated at 4 ^{o}C 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.

Detection of hMPV by cell culture: The cell lines which were used to culture the virus were carcinomic Human type II Alveolar Epithelial (A549). This cell lines were obtained from ATCC (CCL-185). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM). Ten percent of foetal calf serum was 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 patients samples. The infected cells are then incubated in 5% CO_2 incubator, and the cells were observed for CPE up to 14 days (figure - 1).

Immunofluorescence Assay: Immmunofluorescence assay was done by using D^3 DFA Metapneumovirus Identification kit, according to the protocol. This kit is a commercial kit from Diagnostic Hybrids, USA, which is used in this study to detect hMPV antigens by immunofluorescence using a blend of three monoclonal antibodies (MAbs), from patients with signs and symptoms of acute respiratory infection. The limitation of this assay is that it detects the hMPV antigens but is unable to differentiate the four genetic sublineages of the virus. Cells which are to be tested were placed on a glass slide and were 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 apple-green, compared to the uninfected which was stained red by the Evans Blue counter-stain (figure - 2).

Detection of Human Metapneumovirus L gene by PCR: Detection of Human Metapneumovirus L gene was done by conventional Reverse Transcriptase Polymerase Chain reaction**.** The detection of this gene was done from primers obtained from NCBI hMPV L gene (AY550175). The primers were designed using PrimerQuest software. The primers used are, L F (5'-CAC TAT AAA AGT CGA AAA ACA G -3') and L R (5-' CAA TGC ATA GAA AAT AAT TTT C- 3'), which amplified 170bp fragment. Viral RNA was extracted from patient's samples using the Qiagen QIAamp Viral Mini kit (Germany) according to the manufacturer's protocol. Human Metapneumovirus was identified in specimens by using reverse transcription polymerase reaction (RT-PCR). Viral RNA was amplified using the One-step RT-PCR (Qiagen, Germany) which contained 5 µl of 5X OneStep RT-PCR Buffer, 400 µM each dNTP, 0.6 µM each primer, 1 µl of OneStep RT-PCR enzyme mix and 5 µl of extracted RNA in a final volume of 25 µl. RT-PCR parameters comprised 50^{o}C for 30 min and 95^{o}C for 15 min, followed by 40 cycles at 94^{o}C for 1 min, 50^{o}C for 1 min and 72^{o}C for 1 min with a final extension step at 72^{o}C for 10 min. The RT-PCR product was visualized on a UV transilluminator following agarose gel electrophoresis.

Detection of Human Metapneumovirus F gene by PCR: A 450 bp fragment of the hMPV fusion (F) gene was amplified with primers MPVF1 For: (5'-CTTTGGACTTAATGACAGATG-3') and hMPVF2 Rev: (5'-GTCTTCCTGTGCTAACTTTG- 3'), which corresponds from the nucleotide 3704– 4153 [11] All 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).

Phylogenetic Tree construction: Nucleotide sequence obtained from the sequenced analysis was aligned with the sequences obtained from the GeneBank. The multiple sequence alignment was done using CustalW alignment program of Mega software, version 5.03[17]. The average pairwise Jukes–Cantor distance was found to be 0.697 indicating that the data was suitable to construct Neighbour-Joining trees[18]. Trees were constructed using the p-distance nucleotide substitution model, with 1000 bootstrap replicates, using the Mega 5.03 software.

Results and Discussion

Viruses which frequently associated with respiratory infections in humans are rhinoviruses, coronaviruses, influenza viruses, parainfluenza viruses, hRSV, and adenoviruses[19]. Recently, Human metapneumovirus has also known to be a pathogen causing seasonal outbreaks of respiratory infections in children and adults[20].

The Virology unit, of the Institute for Medical Research, Kuala Lumpur received a total of 2600 respiratory samples. These samples were obtained from patients admitted in the hospital for various respiratory illnesses from March 2010 till end of March 2012. From this sample size, 625 patient samples which were negative for RSV, Adenovirus, Parainfluenza 1, 2, 3, Flu A and Flu B which are the routine respiratory viruses detection done in the laboratory of the department.

Based on the detection of hMPV by viral culture and immunofluorescence method, out of the total number of respiratory samples which were cultured, only 131 respiratory samples (20.8%) were positive for hMPV. The observation for CPE for each hMPV isolates ranges from 9 to 14 days of incubation. A higher number of positive hMPV samples were detected by Reverse-Transcriptase PCR, which are about 167 respiratory samples (26.7%). The reason which might have contributed to the negative results would be the acute phase. Viral RNA would decrease when the antibodies in the body appears. Most patient samples were sent for laboratory testing when the treatment had started[21]. This might contribute to the reason in the difference of the number of positive hMPV in the same respiratory sample but different procedure. PCR method is a fast, reliable and cheapest way for viral diagnostic. The method also detects directly into the viral DNA instead of searching for antibodies against the viruses which are produced by the body[22]. He concluded that the RT-PCR test is the best diagnostic method for detecting hMPV in patients.

In this study, samples were taken from patients who presented fever for 2 days and above and required hospitalization. The most common clinical features present on patients with hMPV infection were temperature of ≥37.5, cough, rhinorhoea, wheezing, sore throat, lethargy and shortness of breath (table-1). All 167 patients detected with hMPV infection, experienced three or more symptoms. Most patients admitted were diagnosed having fever (167, 100%), cough (165, 98.8 %), Rhinorhoea (155, 92.8%), wheezing (157, 94.0%), sore throat (141, 84.4%), lethargy (163, 97.6%) and shortness of breath (148, 88.6%). The symptoms observed are similar to other respiratory diseases. The mean duration of hospitalization among hMPV positive cases were 7.5 days ($p < 0.015$) (table - 1).

According to a research done in Canada by Biovin et al., hMPV infected children were hospitalized for <7 days. However, none of the children admitted for hMPV infection were placed in the intensive-care unit [23]. Symptoms like this are common among patients with upper and lower respiratory tract infection which makes it harder to be diagnosed without proper lab testing[24].

Table-1
Symptoms experienced in patient with Human Metapneumovirus infection, February 2010-March 2012

Syptoms	Number of patients with symptoms n=167 (%)
Fever, ≥37.5 (≤ 2 days)	167 (100)
Cough	165(98.8)
Rhinorhoea	155 (92.8)
Wheezing	157 (94.0)
Sore throat	141 (84.4)
Lethargy	163 (97.6)
Shortness of breath	148(88.6)

Although, the incidence of human metapneumovirus infection occurs all year round, most samples received were detected positive during the monsoon season, between May to September 54 cases (32.3%) and from November to March with 98 cases (58.7%). The incidence of human metapneumovirus positive cases was in the highest peak during the month of December with 73 (43.7%) cases. This is probably due to the temperature in Malaysia would be lower due to heavy rain. This would favor the infection of common cold due to decrease of immunity[25].

Mode of Transmission: A virus enters the host through many ways, such as, through the skin, mucosa, mucus membrane, lungs, gastrointestinal tract and genitourinary tract. It is also possible for a virus to enter fetus through the mother's placenta. Currently, the mode of transmission of hMPV have been reported by direct or close contact with the respiratory secretions of patient infected with hMPV and also by contact with objects and surfaces contaminated by their secretions. In a study done by ML von Linstow et al., 2006, hMPV have been detected positive in saliva and sweat sample in patient tested in 1-4 days from the diagnostic time. Out of the 723 patient samples collected, which were positive for hMPV, 14% was detected from saliva and 29% was detected from sweat. The author also indicated that, 17% of patients with hMPV positive had problems related to gastrointestinal tract which include 100% of these patients having anorexia, 17% had vomiting and 33% had diarrhea. In this study, the author could not detect any positive hMPV from urine and blood samples[26].

Phylogenetic analysis: Based on the phylogenetic tree, the data shows well separated groups of all 4 subtypes with patients having infection with hMPV A1 (47), hMPV A2 (58) hMPV B1(31) and hMPV B2 (31). Out of the 167 patient samples that were tested, hMPV A2 was the highest number which contributes to 58 patients, whereas the overall total of the other subtypes, hMPVA1, hMPVB1 and hMPVB2 is 109. This reveals that hMPV A2 prevalence occurred.

The similarity of the sequence was accordance with the topology of the tree. The phylogenetic analysis was constructed based on the F gene of hMPV. The nucleotide identity between groups A and B is 87 %–89 %, the nucleotide identity between subgroup A1–A2 and B1–B2, is 96%–97% and 98.2%–99.7% between subgroups A1–A2 and B1–B2, respectively. Based on the phylogenetic tree, subgroup A2 was the most divergent. The similarity of the F gene sequence shared a (92.8%–99.6%) nucleotide identity (figure-3).

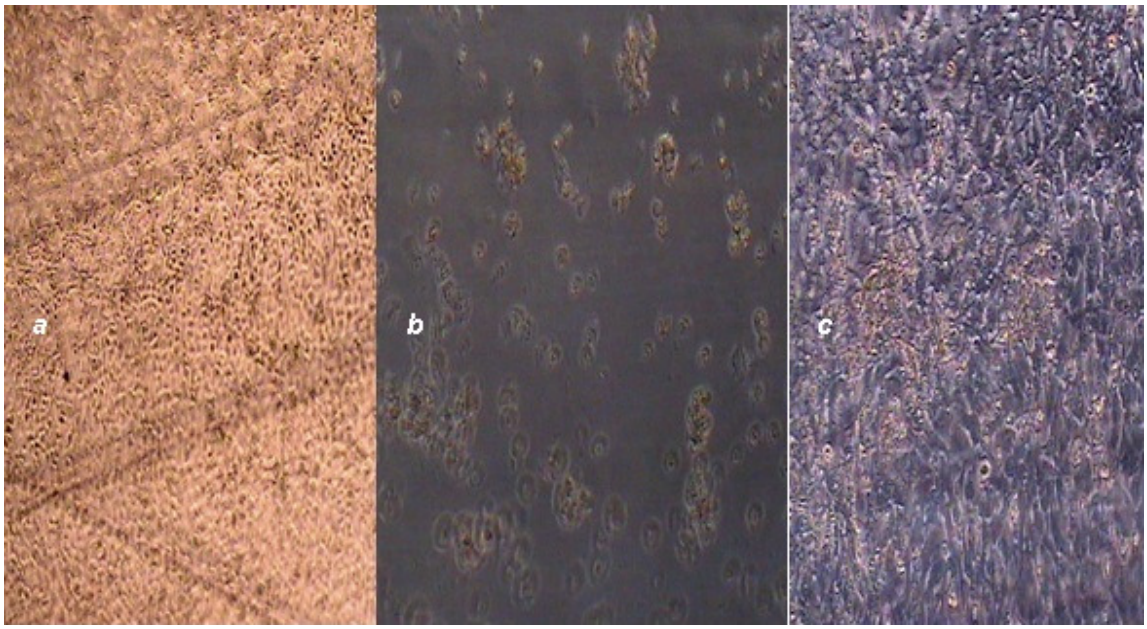

Figure-1
Detection of hMPV by viral culture (a) Control A549 cells-day 1, (b) A549 cells infected with hMPV day 14; (c) Control A549 cells-day 14

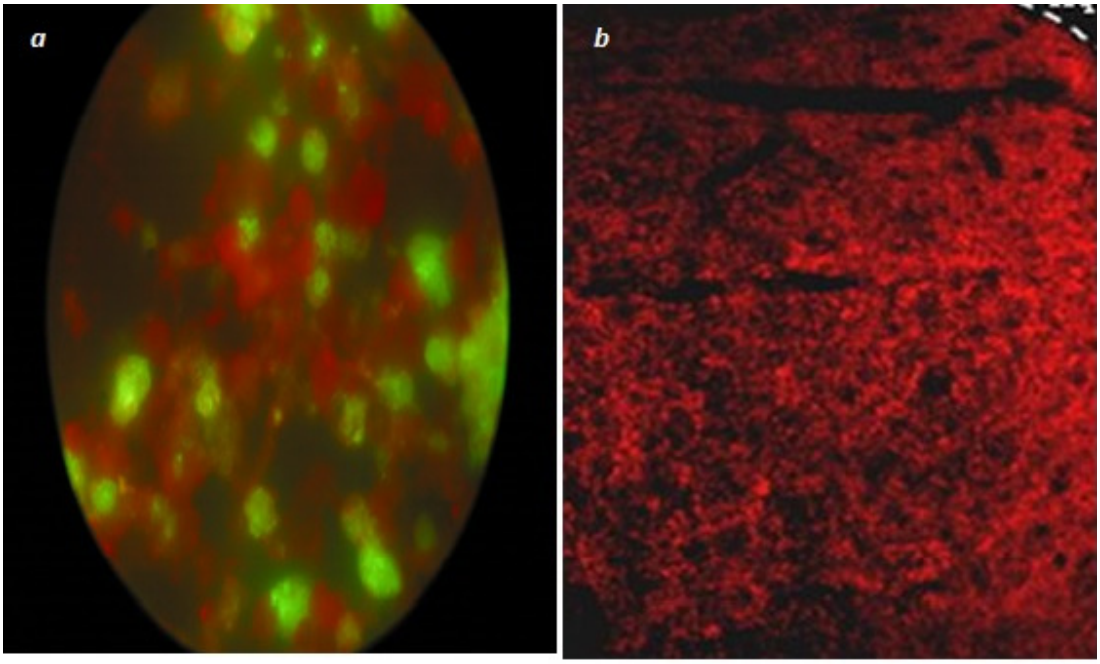

Figure-2
Detection of hMPV using IF staining (a): The infected cells showed an apple green color compared to the uninfected (b), which showed no fluorescence instead stained in red due to the Evans Blue counter stain

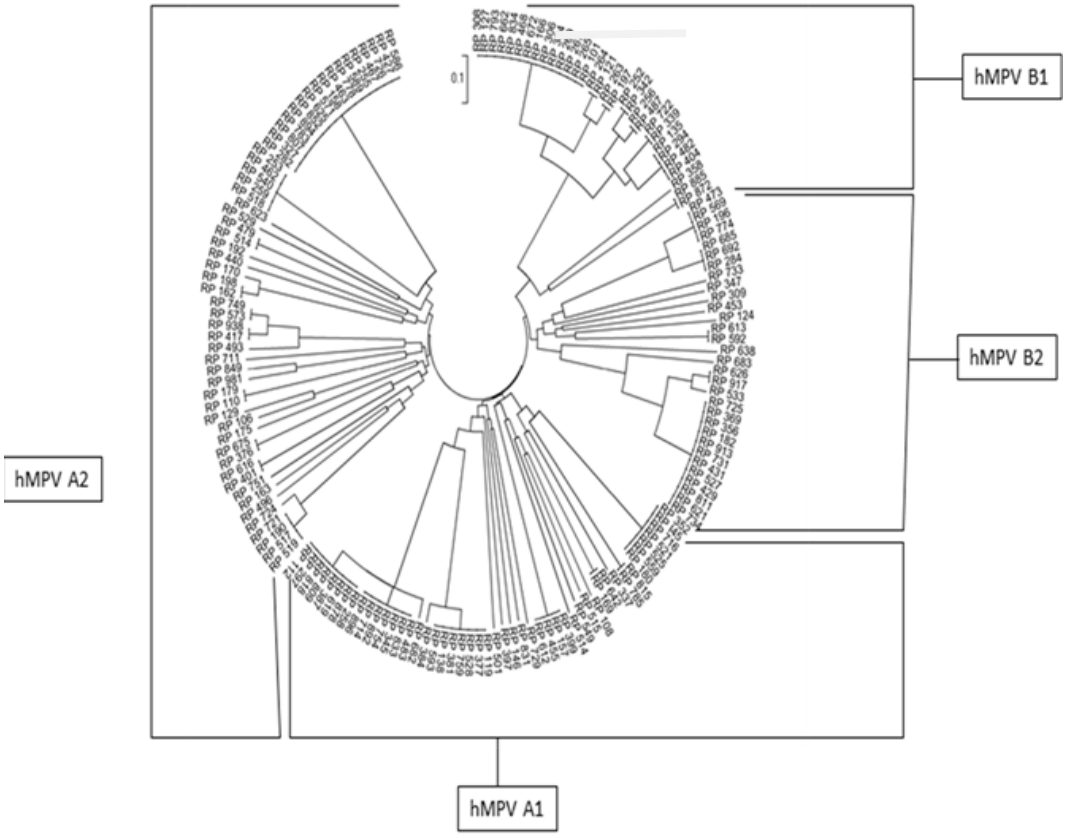

Figure-3
Phylogenetic tree was constructed for all 167 samples using MEGA 5.03 software

Conclusion

These results, provides evidence of hMPV appears to be important cause of both upper and lower respiratory tract infections in young children, immunocompromised individuals and also in adults. However, due to dual infection with other respiratory virus is very common, additional studies using more sensitive diagnostic tests is needed in order to determine the clinical and economical impact of this pathogen in the Malaysian community.

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