

Detection of Human Metapneumovirus in Hospitalized Children with Acute Respiratory Tract Infections in Sulaimani Province, Iraq

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Abstract

Background: Human Metapneumovirus (hMPV) is a member of Paramyxoviridae family. It is an important viral pathogen of respiratory tract infections among children. Therefore, the aim of this study is to determine the incidence of hMPV infections among children less than 5 years of age with respiratory tract infections at the pediatric hospital in Sulaimani city of Iraq. Also, this study aims to evaluate the different diagnostic methods for the detection of this virus.

Method: Nasopharyngeal swabs and throat swabs were collected from 300 hospitalized children with respiratory infections under 5 years of age between April 2011 and March 2012. Each sample was used for hMPV detection by conventional reverse transcriptase (RT-PCR) and direct fluorescent assay (DFA). A questionnaire which was designed for acute respiratory tract infections among hospitalized children was also included.

Results: Human Metapneumovirus was detected in 16% by RT-PCR and 14.7% by DFA. Autumn – winter period was the most common season for hMPV infections with its peak occurrence in January and February.

Conclusions: hMPV is an important pathogen associated with RTIs in children. RT-PCR is highly sensitive and specific for the detection of hMPV than antigen detection methods used for the diagnosis of hMPV viruses.

Keywords: Human metapneumovirus; Hospitalized children; Direct fluorescent assay; RT-PCR

Introduction

Human Metapneumovirus (hMPV) is an enveloped single stranded negative-sense RNA virus [1]. It is a member of the *Paramyxoviridae* family, subfamily *Pneumovirinae*, and genus *Metapneumovirus* [2]. The hMPV was first identified in Netherland by Van den Hoogen, et al. However, the virus was first isolated from stored nasopharyngeal aspirates of infected children collected over a 20 year period [3].

hMPV appears to have respiratory epithelium tropism [4]. The symptoms of both upper and lower respiratory tract disease have been associated with hMPV infections in infants, young children, elderly, and immunocompromized patients. However, at the age of five years, virtually every individual has experienced at least one hMPV infection [5-7]. In young hospitalized children, the clinical features associated with hMPV infections are similar to those of RSV [8].

There are mainly two major techniques for the detection of hMPV from respiratory specimens, conventional reverse transcriptase – PCR (RT-PCR) and immunofluorescence technique, although shell vial culture and routine tissue culture can also be use [9,10].

The laboratory diagnostic methods that are routinely used in our hospitals do not usually include the diagnosis of hMPV in their schedule investigations. Therefore, to the best of our knowledge, data on the incidence and clinical importance of hMPV infections in children with ARTI in Sulaimani province of Iraq is limited.

This study aims to determine the epidemiological characteristics of hMPV infections among hospitalized children with ARTI and immunocompromized children from Hiwa hospital in Sulaimani province of Iraq.

Materials and Methods

Study design

Cross sectional, hospital – based study.

Patients

Three hundred hospitalized children were involved in this study: two hundred and seventy hospitalized children less than five years of age, were admitted to Pediatric Teaching Hospital in Sulaimani city for respiratory tract infections of undiagnosed etiology, while thirty children were immunocompromized, suffering from RTIs in Hiwa Hospital. Both genders were included and were chosen by random sampling. The specimen collection began from April 2011 to March 2012. In addition, a signed informed consent was obtained from the legal guardians of the children who participated in this study.

Questionnaire

The patients were subjected to a questionnaire that included demographical and clinical data related to respiratory infections.

Specimens

Two Nasopharyngeal swabs (NPSs) were taken from each child by flocked swab applicators (Micro Rheologics Company/Italy). One NPS was placed immediately after collection in 3 ml viral transport medium (VTM), and then VTM was stored at -70°C. The other swab was immediately rolled on the labeled slide. In addition to NPSs, two throat swabs (TSs) were also collected from each child, and these TSs were manipulated in the same manner as NPSs.

Methods

Both direct immunofluorescence assay (DFA) and conventional

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reverse transcriptase – PCR (RT-PCR) were used for the detection of hMPV from the swabs collected from the children in this study.

DFA

DFA was performed according to the manufacturer’s instructions (Light Diagnostics, Millipore, USA). In summary, the nasopharyngeal swab was rolled on the slide, and the epithelial cells were fixed in cold 100% acetone for 10 minutes at 2-8°C. The spot of specimen on slide was bordered by a marker pen. Thus, about 40 µl of SIMULFLUOR HMPV/RSV reagent (conjugate) was used to cover the spot of the smear. The slide was incubated for 15 minutes at 37°C in a humid chamber, and then it was rinsed thoroughly with PBS/Tween 20 solution, and allowed to air dry. The slide was then mounted under a cover slip using an aqueous mounting medium. Microscopical examination of slide was performed using a fluorescence microscope (Olympus BX60; Olympus Optical Co. Ltd., Tokyo, Japan) with 100-400x magnification for cells exhibiting the apple green fluorescence of fluorescein isothiocyanate (FITC) stain for hMPV positive slides.

Molecular technique

The conventional reverse transcriptase-PCR (RT-PCR) was the molecular technique for the detection of hMPV. The technique targeted the F gene of hMPV genome using primers designed in this study (Table 1). However, it was taken from NCBI Reference Sequence: NC_004148.2.

Therefore, the technique was performed in the following sequence:

1. Extraction of viral RNA from NPS using viral DNA/RNA extraction kit (Intron Biotechnology Company/South Korea), and was performed according to the manufacturer’s instruction.

2. Reverse transcription was done with two-steps reverse transcriptase-PCR kit in thermal cycler (TC312/ Techne Company/ United Kingdom) for the synthesis of first strand cDNA; and it was performed according to the manufacturer’s instruction.

3. PCR amplification: The PCR amplification protocol which is mentioned in Table 2, was used in this study. Also, ready to use positive control was used to check the third step.

4. Horizontal gel electrophoresis (Phero – Sub 1010-E/ Biotec – Fischer Company/USA) was used for the detection of PCR products and the amplicons were verified using DNA ladder, and then, the amplicons were viewed in Gel documentation (Uvido/ Uvitec Company/UK). The components were added in one micro centrifuge tube for a total of 25 µl reaction volume. Positive control is added in a separate tube with its primers.

Gene	Polarity of primer	Nucleotide position of primer	Primer sequence (5' → 3')	Amplicon size
hMPV F gene	F	3796-3815	ATGTTGGAGAACCGTGCGAT	465
	R	4260-4241	CCCTACTCTGTTGCTGCCAA	

Table 1: Primers used in this study.

Component	Volume
Master mix	12.5 µl
Forward primers	3 µl
Reverse primers	3 µl
CDNA	6.5 µl

Table 2: The protocol for PCR amplification.

Results

The DFA technique for the detection of hMPV antigen in epithelial cells of nasopharynx showed that positive specimens were emitting green fluorescence as they were stained with fluorescein isothiocyanate (FITC) dye, and were examined under a fluorescent microscope (Figures 1 and 2). The results revealed that 44 (14.7%) NPS specimens were hMPV positive, while the remaining 256 (85.3%) patients were negative. Also, the results of the TS specimens revealed 41 (13.7%) positive and 259 (86.7%) negative cases. Therefore, the DFA results showed that the differences between NPS and TS were not statistically significant (P=0.8150) (Figure 3).

Conventional RT-PCR was performed for the detection of hMPV from the collected specimens. The results revealed that 48 (16%) patients were positive, while the remaining 252 (84%) were negative.

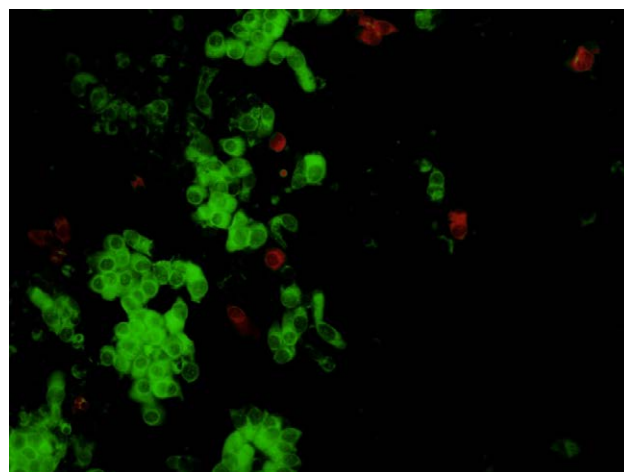


Figure 1: Positive result of DFA technique seen under a fluorescent microscope for the detection of hMPV antigen in nasopharyngeal swab using FITC stain. The pseudostratified columnar epithelial cells of the nasopharynx, shows prominent green fluorescence in cytoplasm (arrow), while the nuclear region is pale.

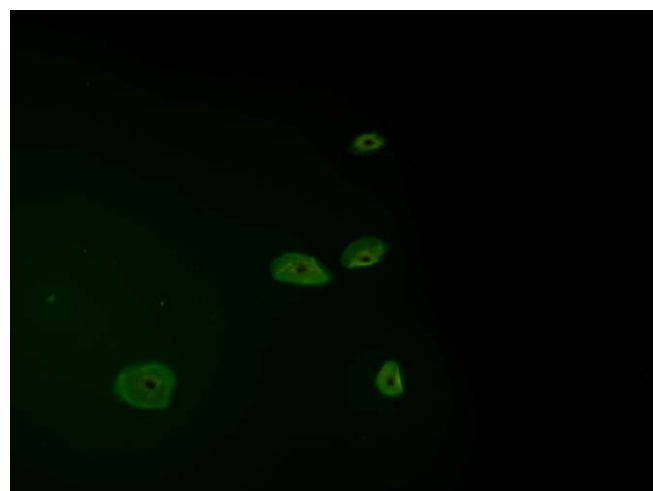


Figure 2: Positive result of DFA technique seen under a fluorescent microscope for the detection of the hMPV antigen in the throat swab using FITC stain. The stratified squamous epithelial cells of oropharynx are showing prominent green fluorescence in cytoplasm (arrows).

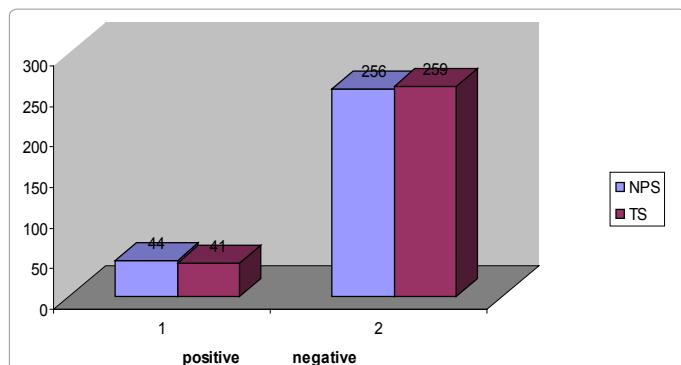


Figure 3: Results of DFA technique for the detection of hMPV in NPS and TS for children in the study.

Patients	Positive cases	Negative cases	Total
Immunocompetent	44	226	270
Immunocompromized	4	26	30
Total	48	252	300

Table 3: Comparison in results of hMPV infections among immunocompromized and immunocompetent patients.

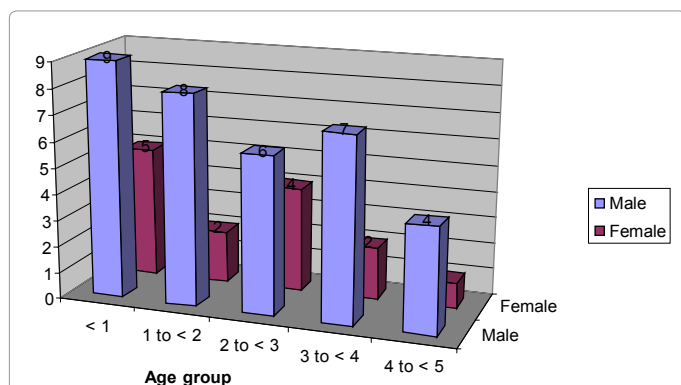


Figure 4: Age and sex distribution of hMPV positive children enrolled in the study.

	Frequency of HMPV positive patients	Frequency of HMPV negative patients	P value
Sex			
Male	34	159	0.3290
Female	14	93	
Kindergarten or nursery presence	4	49	<0.0001
Residency			
City	21	107	0.02761
District	12	103	
Village	15	42	
Clinical features			
Sudden onset	9	55	0.7047
Insidious onset	39	197	
Fever	36	192	0.8549
Dyspnoea	38	188	0.5861
Cough	29	212	0.0005
Sputum	14	73	0.3925
Wheezing	32	127	0.0616
Tachypnoea	4	22	1.0000
Runny nose	26	109	0.2052

Abnormal breathing sounds	40	120	<0.0001
Added breathing sounds	28	88	0.0123
Cyanosis	3	33	0.2300
History of chronic disease	4	31	0.6235
History of previous similar condition	23	125	0.2650
History of contact with similar condition	16	31	0.0030
Family history of chronic respiratory disease	3	21	0.2698
Mean hospital stay (days)	4.0	3.3	0.1168
BMI			
Normal	41	213	0.1654
Above normal	1	21	
Below normal	6	18	
WBCs count			
High total	42	165	0.0020
Granulocytosis	29	108	0.7178
Lymphocytosis	10	48	0.5674
Clinical illness			
Bronchiolitis	13	66	0.0078
Pneumonia	21	60	
Others	14	126	
SpO₂ (%)			
Normal	8	130	<0.0001
Subnormal	40	114	
Chest x-ray			
Normal	14	145	0.0004
Abnormal	34	107	
Type of treatment			
Supportive	48	252	1.000
Specific antibacterial drugs	48	250	
Specific antiviral drugs	0	0	
Condition at discharge			
Complete recovery	3	19	1.000
Discharged with medication	45	230	
Death	0	3	

Table 4: Demographic and clinical data of HMPV positive patients and HMPV negative patients.

Thus, each reaction tube for each patient represents a combination of NPS and TS specimens.

All hospitalized immunocompromized patients were tested for hMPV infections, and the results showed that 4 (13.3%) patients from the total number of 30 patients were positive, while the remaining 26 (86.7%) were negative for hMPV as tested by RT-PCR. The same results were also obtained when specimens were tested by DFA. Hence, the differences in results between the immunocompromized and immunocompetent patients are not statistically significant (p=0.7983) (Table 3).

The total number of hospitalized children that participated in this study was 300, and all of them were under 5 years of age. 48 (16%) were positive for hMPV when tested by RT-PCR, and 252 (84%) were negative. The 48 hMPV positive patients were mostly males and were 34 (70%), whereas 14 (29.2%) of them were females. Hence, the male to female ratio was 2.4:1.

All children were divided into five groups, <1, 1 to <2, 2 to <3, 3 to <4, and 4 to <5. The highest hMPV infection detected by RT-PCR was in the age group <1 years old, while the lowest frequency was in

age group 4 to <5 years. Thus, the age and sex distribution of hMPV is described in Figure 4. Demographic and clinical characteristics of hMPV infections among children in this study are summarized in Table 4. Some significant differences were observed among demographic data. With the exception of few features, the clinical features showed no significant statistical differences between hMPV positive and hMPV negative patients.

Pneumonia and bronchiolitis were more frequent among hMPV positive patients than other group (P value <0.05).

The results of this study showed that the peak of hMPV infections was during February, which was 9 out of 48 (18.8%) hMPV positive patients, while the lowest frequency of infections was present in July which was negative for any hMPV infections. Therefore, autumn and winter is the highest season of hMPV infections, while summer is the least season of hMPV infections (Figure 5).

The calculation of sensitivity, specificity, and predictive values was performed for DFA depending on RT-PCR as a standard test. The results showed DFA sensitivity of 91.7%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 98.4% (Table 5).

Discussion

To the best of our knowledge, this is the first report for the detection of hMPV infection in Iraq using RT-PCR compared to Immunofluorescent technique. Seroprevalence of IgM and IgG anti-hMPV antibodies among hospitalized children under 5 years of age in Sulaimani city were 12% and 75%, respectively [5]. Consequently, human Metapneumovirus has shown to be responsible for a significant number of ARTIs in early infancy and childhood (16%), and in immunocompromized children (14.7%) in this study. RT-PCR was highly sensitive and specific for the detection of hMPV, making it a valuable and a competitive technique with immunofluorescent assay for the detection of the virus. The frequency of hMPV infections highlighted by this study showed some variations with those that have

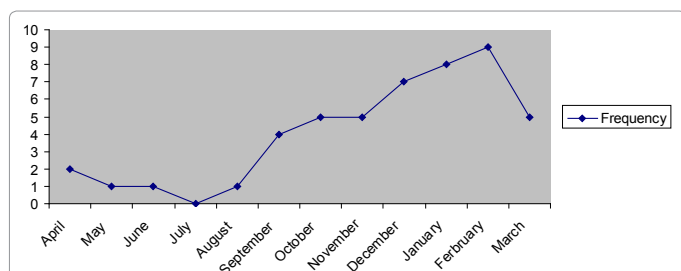


Figure 5: Seasonal distributions of hMPV infections among hospitalized children in the study group.

Parameter	DFA	RT-PCR
True positive	44	48
False positive	0	0
True negative	252	252
False negative	4	0
Total number	300	300
Sensitivity	91.7%	100%
Specificity	100%	100%
Positive predictive value	100%	100%
Negative predictive value	98.4%	100%

Table 5: Sensitivity, specificity, and predictive values for the detection of hMPV in respiratory specimens.

emerged from previously published studies in different countries [11-19]. These variations in incidence among studies might reflect different epidemiological patterns of hMPV infections in different countries, which in turn might be related to environmental factors, geographical factors, differences in host genetic susceptibility, sampling techniques, detection methods, and/or different viral strains circulating in different geographical areas.

The use of PCR was particularly advantageous for hMPV, because the virus is fastidious and difficult to grow in most cell lines [20]. The sensitivity and specificity of DFA for the detection of hMPV were very similar to the results of Zhang, et al. When compared with RT-PCR, the DFA is a rapid and simple alternative technique, thus facilitating rapid diagnosis [21].

Fever, dyspnoea, wheezing, abnormal breathing sounds, and added sounds were the most frequent clinical features among hMPV positive children, while Ji Wang, et al. reported fever, cough, and rhinorrhea as the main clinical manifestation [22]. This might reflect the broad spectrum of clinical diseases with different manifestations in hMPV infections.

The general investigations showed that leukocytosis, subnormal SpO₂%, and abnormal chest x-ray were present more frequently in hMPV positive children than in the other group. Hence, the differences between the two groups were statistically significant (Table 4). These findings show that hMPV infections are moderate to severe in potency.

Pneumonia and bronchiolitis were the main clinical diseases (70.8%) in hMPV positive children with 43.8% and 27%, respectively. Wei et al. reported bronchiolitis as the main clinical disease [19], while another study recorded pneumonia as the main clinical disease [23].

The study showed that hMPV infections were higher among age group <1 year (29.2%) when compared to older children. This result was comparable to that of Chen, et al. [24], Sohier Yahia, et al. [16], and Cui Zhang, et al. [25]. The higher incidence of hMPV infections among age group less than one year, might signify the importance of this age group as a risk factor for infection. However, Papenburg, et al. reported comparable results that children <6 months are at risk of hMPV infections [26].

In hMPV positive children, the male to female ratio was 2.4:1. Thus, this indicates male predominance, but the result was not significant when compared to hMPV negative children. Most published studies reported no significant gender predominance; Pilger, et al. recorded results similar to the current study [27], Zuo, et al. showed no sex difference among hMPV positive children [28], while Rein, et al. found that 53.2% of children infected with HMPV were females [29].

The results showed that 4 (13.3%) out of 30 of immunocompromized children were hMPV positive, while the remaining 26 (86.7%) were hMPV negative by DFA and RT-PCR. Therefore, the results were not statistically significant when compared to immunocompetent children. The clinical features of hMPV among the two groups showed no differences. Similar results were also obtained by John Williams et al., who found that patients infected with hMPV had varying degrees of immunosuppression, thereby suggesting that severe immune suppression is not a necessary risk factor for hMPV infection [30].

The results also clarified that living in rural regions was more in hMPV positive children than in the other group, and contact with respiratory infected patients was more frequent among hMPV positive children than in the second group. Comparable results were also found in other studies [31,32].

The sample collection was done between April 2011 to March 2012, and the majority of hMPV positive patients were detected during the autumn and winter period with a peak in February. Other studies reported variable seasonal predominance. In a study done in China, spring was the main season [33]; in Jordan, the peak was reported in March [13]; in India, HMPV infection rate was at its peak in spring-summer period of 2008-2009 and 2009-2010; and hMPV circulated predominantly during the winter-spring period of 2010-2011 [34].

Conclusion

The hMPV is an important cause of RTIs for hospitalized children in Sulaimani city, and both RT-PCR and DFA are valuable diagnostic techniques for this virus. However, RT-PCR is more sensitive than DFA. In addition, the autumn and winter period was the most frequent seasons for hMPV infections.

Abbreviations

Human Metapneumovirus (hMPV), respiratory tract infections (RTIs), viral transport medium (VTM), Nasopharyngeal swabs (NPSs), throat swabs (TSs), direct immunofluorescence assay (DFA), conventional reverse transcriptase – PCR (RT-PCR), Respiratory syncytial virus (RSV).

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