



International Journal of  
**Virology**

ISSN 1816-4900



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## **Detection of Human Metapneumo Virus among Infants with Bronchiolitis**

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### **ABSTRACT**

Human Metapneumo Virus (hMPV) is a major cause of lower respiratory tract infections in infants, elderly and immune-compromised patients. It is the second viral etiological agent, after RSV which causes Respiratory Tract Infections (RTI) in children, especially children below 5 years old. hMPV isolation in cell culture is trypsin-dependent and requires long incubation times and untypical cell lines. Antibody responses have not been useful in the diagnoses of acute hMPV infections. Commercial kits for detection of hMPV antigens by monoclonal antibodies in clinical samples have become available, promoting the rapid detection of the virus. The present study aimed to detect human metapneumo virus in infants with suspected viral chest infection and compare Direct Fluorescent (DFA) with nested RT-PCR assay in detection of the virus. This study included 30 children <18 months presented to pediatric emergency room of Ain Shams University Children's Hospital diagnosed clinically as cases of bronchiolitis. Nasopharyngeal aspirates were collected and transferred to the lab on calf bovine serum to be examined by Direct Immune-Fluorescence (DFA) for presence of hMPV antigen and nested-RT PCR for detection of hMPV genome. Six out of thirty samples included in the study were positive for hMPV antigen by DFA and three out of thirty were positive for hMPV gene by RT-PCR assay with 100% sensitivity and 89% specificity for DFA. Human metapneumo virus infection is a leading cause of respiratory tract infection in Infants; DFA can be used as a primary and easy diagnostic test for detection of hMPV in those infants.

**Key words:** Human metapneumo virus, direct immunofluorescent assay (DFA), RT-PCR, bronchiolitis

### **INTRODUCTION**

Human metapneumo virus (hMPV) is one of the latest discovered viruses. It belongs to Paramyxoviridae family. It was first identified as a major cause of respiratory infections in Netherlands (Van den Hoogen *et al.*, 2001). It is the second viral etiological agent, after Respiratory Syncytial Virus (RSV) which causes Respiratory Tract Infections (RTI) in children, especially children below 5 years old. It is estimated that 5-25% of RTI in children is due to hMPV (Kahn, 2006; Williams *et al.*, 2004). Seroprevalence studies revealed that 25% of all children aged 6-12 months who were tested in the Netherlands had detectable antibodies to human metapneumovirus; by age 5 years, 100% of patients showed evidence of past infection. Separate reports from all over the world support the early contention that this virus is ubiquitous and like RSV, is seasonal in

nature. In addition, a Chinese study showed winter preference of viral infection as the virion particles are more virulent when ambient temperature was fairly low. The incidence of hMPV infection was negatively correlated with the average monthly temperature and rainfall. So, they demonstrated that hMPV infection occurred throughout the year with peaks during late winter and early spring so the climatic factors, especially monthly average temperature, may affect the prevalence of hMPV in different areas of the world. In infants and children, lower respiratory tract illness caused by hMPV is similar to other viral associated bronchiolitis and cannot clinically be distinguished from the RSV, influenza and Parainfluenza viruses. Cough was reported in more than 90% of children; more than 75% had rhinorrhea and more than 50% had fever (Biacchesi *et al.*, 2003; Williams *et al.*, 2004). Hypoxia and abnormal chest radiograph findings were common in hospitalized patients; most infants and children received a discharge diagnosis of bronchiolitis, croup, pneumonia, bronchitis, or asthma exacerbation (in decreasing order of frequency) (Alvarez and Tripp, 2005). Also, Bacterial otitis media is a common complication of hMPV upper respiratory tract infection resulting from Eustachian tube dysfunction followed by bacterial stasis in the middle ear. hMPV antigens and nucleic acids were reported in middle ear fluids. Co-infection with other respiratory viruses can occur and simultaneous infection with hMPV and RSV has been postulated to cause severe disease, often requiring intensive care admission and ventilatory support (Mullins *et al.*, 2004). The disease is believed to be more serious in infants and in those with chronic illnesses (Williams *et al.*, 2005).

Despite the universal infection in childhood, new infections can occur throughout life due to incompletely protective immune responses and/or acquisition of new genotype. Since severe disease is seen mainly in pediatric patients, it suggests that naturally acquired infection induces partial protection against the disease. An important clinical correlation between hMPV infection and COPD was demonstrated by many studies, they yielded a controversial view for the role of hMPV in exacerbation of COPDs. hMPV can also cause severe or fatal disease in patients with malignancy or hemopoietic cell transplants (Collins and Crowe, 2007).

Laboratory diagnosis of hMPV infection is the corner stone for definitive verification of clinical diagnosis. Early diagnosis is advantageous because it allows implementation of isolation measures to limit spread in gathering communities as hospital setting or nursing home (Collins and Crowe, 2007). However the diagnosis of hMPV infection is problematic, as the virus is difficult to isolate from routine cell culture lines, RT-PCR examination of respiratory secretions is currently the clinical test of choice to reliably diagnose hMPV infection (Van den Hoogen *et al.*, 2004). Yet there is currently no consensus on the optimal primer set for hMPV detection. As the sensitivity of RT-PCR may vary depending on which primer set is selected or which gene is targeted (Landry *et al.*, 2005). An indirect immunofluorescent antibody test was developed that allows serologic diagnosis. With the virus identified and a serologic test available. However, monoclonal antibodies have been described which are able to detect hMPV in nasopharyngeal aspirates and the concept of a traditional immunofluorescent assay has now been proven (Percivalle *et al.*, 2005). A direct immunofluorescence test utilizing specific monoclonal antibodies offers a rapid, sensitive and specific method for direct detection of hMPV in clinical samples such as nasopharyngeal aspirates (IMAGEN™ hMPV, 2009). The overlapping seasonal occurrence of hRSV and hMPV with other respiratory viruses (Williams *et al.*, 2004) and the inability to clinically distinguish between RSV and hMPV infections or illnesses seen with other respiratory viruses emphasize the need for a sensitive and rapid detection of these viruses (Wolf *et al.*, 2006).

**Aim of the work:** This study aimed to detect hMPV in nasopharyngeal aspirate among infant with clinical diagnosis of bronchiolitis and compare direct fluorescent assay (DFA) with RT-PCR assay in viral detection.

**Patients and methods:** This study included 30 children <18 months presented to pediatric emergency room of Ain Shams University Children's Hospital (a tertiary care centre) and diagnosed as bronchiolitis based on clinical symptoms and signs (rhinitis, tachypnea, wheezing, cough, crackles, use of accessory muscles and/or nasal flaring) according to the Lieberthal *et al.* (2006) position statement, Children with other systemic complaints or history of bronchial asthma were excluded. All studies were conducted with the approval of the Medical ethical Committee, Faculty of Medicine, Ain Shams University. Parents approved the participation in the study by an informed consent, nasopharyngeal aspirates were collected and transferred to the lab on calf bovine serum to be examined by Direct immunofluorescence for presence of hMPV antigen and nested-RT PCR for detection of HMPV genome.

#### **DIRECT IMMUNOFLUORESCENCE TEST**

**Principle of the test:** The IMAGEN™ hMPV test contains monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC). The conjugated antibodies bind specifically to viral antigens present in all strains of hMPV. The reagent is used in a one-step direct immunofluorescence technique. The collected nasopharyngeal aspirates were transferred to the lab on calf bovine serum. According to the manufacturer instructions, specimens were incubated with the reagent containing FITC conjugated antibodies for 15 min, then excess reagent is washed off with Phosphate Buffered Saline (PBS). The stained area is mounted and viewed microscopically using epifluorescent illumination. If hMPV antigen is present, characteristic bright apple green fluorescence is seen within infected cells which contrasts with the red background staining of uninfected cells.

**Technique of the test :** Cell separation was done through adding 2 mL Phosphate Buffer Saline (PBS) before centrifugation to reduce viscosity and dilute the mucus. the mucus extractor was centrifuged at room temperature (15-30°C) for 10 min at 380 g. Remove the supernatant then, the cell deposit was suspended in 2 mL PBS and gently the cells were pipetted up and down with a wide pore pipette, or vortexed gently, until the mucus is broken up and cellular material released. removing the supernatant and suspend the cell deposit in 2 mL PBS and gently pipette the cell up and down until mucus is broken up and cellular material released. Excess mucus was removed as it prevents adequate penetration of the reagent and results in non-specific fluorescence.

**Preparation of slides:** The resultant cell suspension was centrifuged at room temperature (15-30°C) for 10 min at 380 g and discard the supernatant. The cell deposit was re-suspended in sufficient PBS to dilute any remaining mucus while at the same time maintaining a high cell density. The 25 µL of the re-suspended cell deposit was placed into a 6 mm well area on the slide. (The remainder of the specimen was kept at -70 for further PCR procedure). The specimen was left to air dry thoroughly at room temperature (15-30°C) and fixed in fresh acetone at room temperature (15-30°C) for 10 min.

**Staining procedure:** The 25 µL of IMAGEN™ hMPV reagent were added to the fixed cell preparation on the slide or to a Positive Control Slide ensuring that the reagent covers the entire well area.

**First incubation:** The slides with the reagent were incubated in a moist chamber for 15 min at 37°C ensuring that reagent was not dry on the specimen, as this causes the appearance of non-specific staining.

**Washing:** Excess reagent was washed off with Phosphate Buffered Saline (PBS) then slide was washed off in an agitating bath containing PBS for 5 min. PBS was drained off and the slide was allowed to air dry at room temperature (15-30°C).

**Addition of mounting fluid:** One drop of IMAGEN™ hMPV Mounting Fluid was added to the centre of each well and a cover slip was placed over the Mounting Fluid and specimen ensuring that no air bubbles were trapped.

**Reading and interpretation:** The entire well containing the stained specimen was examined using an epifluorescence microscope. Fluorescence was visible at×400 magnification. A positive diagnosis was made when one or more cells showed typical fluorescence in the fixed, stained specimen.

**Positive control slide:** Was stained according to the manufacturer instructions, it showed cells with apple green fluorescent intracellular cytoplasmic granules or filaments contrasting against a red background of counterstained specimen. Positive Control Slides were used to check that the staining procedure has been satisfactorily performed.

**Nested reverse transcription-polymerase chain reaction (RT-PCR):** To detect the RNA genome of hMPV, we used a nested RT-PCR focused on the F-gene. After one cycle of freeze and-thaw, RNA was extracted from nasopharyngeal aspirate by using the QIA amp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's protocol. The outer primers were 5'-AGC TGT TCC ATT GGC AGC A-3' for RT and amplification and 5'-ATG CTG TTC

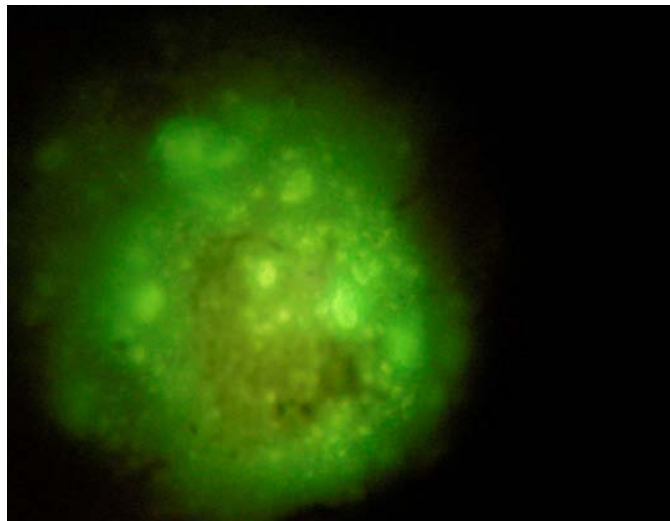


Fig. 1: Positively stained slide preparation showing green apple stained hMPV Ag vs negatively stained red cells

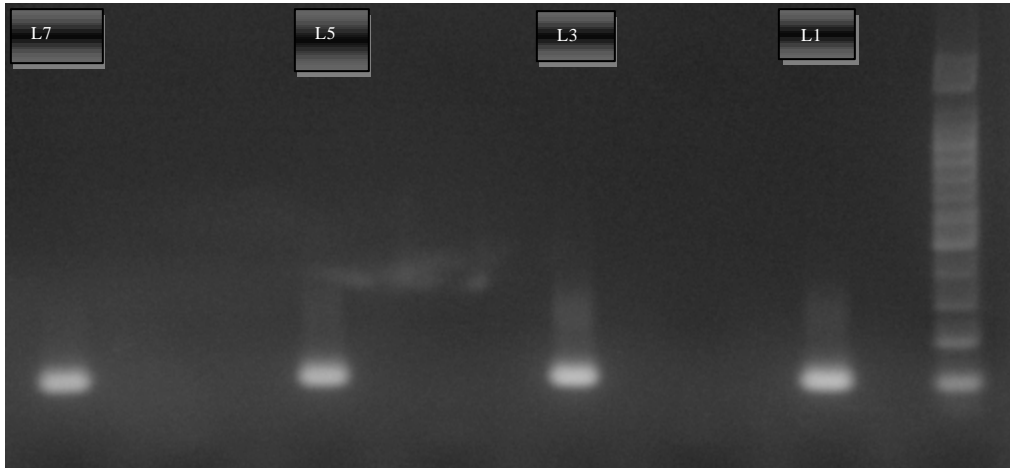


Fig. 2: Ethidium bromide stained agarose gel 2.5% of specific RT-PCR products of hMPV RNA detection; Lane 1 showing a positive control band, Lanes 3, 5 and 7 show positive results for RT-PCR product

RCC YTC AAC TTT-3' (R = A or G, Y = C or T) for amplification. These primers were designed on the basis of hMPV sequences available from GenBank. The reaction was carried out in a single-tube (Superscript One-Step RT-PCR and Platinum Taq; Invitrogen Corp., Carlsbad, CA) by using 0.2 M of each primer and thermal cycling conditions of 50°C for 30 min and 94°C for 3 min; followed by 40 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec and a final extension at 72°C for 7 min. For the second round of amplification, we used 0.2 M of inner primers 5'-GAG TAG GGA TCA TCA AGC A-3' and 5'-GCT TAG CTG RTA TAC AGT GTT-3'. The PCR was conducted at 95°C for 15 min for denaturation of DNA templates and activation of the hot-start DNA polymerase (HotStarTaq, QIAGEN GmbH), followed by 40 cycles at 94°C for 30 sec, 54°C for 30 sec and 72°C for 45 sec, and a final extension at 72°C for 7 min. PCR products detected by agarose gel electrophoresis were analyzed for sequence homology with known hMPV strains (Fig. 1, 2).

## RESULTS

**Statistical analysis:** The collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (SPSS, 2001). Quantitative non parametric variables are expressed as mean and SD, while non parametric was expressed as Median and Interquartile Range (IQR). Qualitative variables are expressed as frequencies and percents. Student t test and Mann Whitney test were used to compare a continuous variable between two study groups. Fisher's exact test was used to examine the relationship between Categorical variables. Kappa statistics was used to compute the measure of agreement between PCR and DFA. A significance level of  $p < 0.05$  was used in all tests.

Six out of thirty samples (20%) included in the study was positive for hMPV antigen by DFA and three out of thirty (10%) were positive for hMPV gene by RT-PCR assay with 100% sensitivity and 89% specificity for DFA (Table 1-4).

Table 1: Description of personal and demographic data among studied cases

Parameter	N	(%)	Mean	±SD	Minimum	Maximum	Median	IQR
<b>Sex</b>								
Male	18	60.0						
Female	12	40.0						
<b>Feeding</b>								
Breast	17	56.7						
Artificial	13	43.3						
Age (months)			7.53	2.57	3.00	14.00		
Temperature			37.56	0.61	36.70	38.80		

Table 2: Description of DFA and PCR results among studied cases

Parameter	N	(%)
<b>DFA</b>		
Negative	24	80.0
Positive	6	20.0
<b>PCR</b>		
Negative	27	90.0
Positive	3	10.0

Table 3: Agreement between PCR and DFA results

Parameter	PCR				Kappa	p	Sig.
	Negative		Positive				
	N	%	N	%			
<b>DFA</b>							
Negative	24	88.9	0	0.0	0.615	0.001	HS
Positive	3	11.1	3	100.0			

Table 4: Value of DFA for diagnosis of HMPV

Parameter	Sensitivity	Specificity	PPV	NPV	Accuracy
DFA	100%	88.9%	50%	100%	90%

## DISCUSSION

Isolation of hMPV is known to be difficult, that is why the virus could not be detected until recently. The first report on hMPV by Van den Hoogen *et al.* (2004) showed that the virus produced syncytia formation in tertiary monkey kidney cells (MDC), followed by rapid internal disruption of the cells and subsequent detachment from cell monolayer. The virus replicated poorly in Vero cells and human lung adenocarcinoma (A-549) cells and could not be propagated in Madin-Darby Canine Kidney (MDCK) cells or chicken embryo fibroblasts (Collins and Crowe, 2007). Human metapneumovirus infection accounts for approximately 2-12% of pediatric lower respiratory illnesses and a lesser percentage in adults (Boivin *et al.*, 2003; Williams *et al.*, 2004). It is interesting to note that detection rates of hMPV have generally been higher in retrospective studies than in prospective studies, an observation consistent with a degree of selection bias. This indicates that large prospective studies are needed in order to clarify the role of hMPV in various clinical conditions (Hamelin *et al.*, 2004).

Identification of hMPV relies on the detection of viral antigens and viral nucleic acids in clinical samples. The use of RT-PCR for the detection of hMPV has been described in several studies and has been found to have greater sensitivity than DFA and viral culture (Collins and Crowe, 2007).

Immunofluorescence is an alternative means to diagnosing hMPV infection, but it has not been adopted for clinical use (Landry *et al.*, 2005; Sumino *et al.*, 2005).

The present work provided also a comparative clue regarding the sensitivity of Direct Fluorescent Assay (DFA) with nested RT-PCR assay. Considering DFA as an example of a diagnostic test used to identify a disease. The specificity of such test is defined as the proportion of patients who do not have the disease who will test negative for it. If a test has high specificity, a positive result from the test means a high probability of the presence of disease. Consequently, a highly specific test, as DFA test is unlikely to give a false positive result. In the present study; the DFA detected six out of thirty samples positive for hMPV antigen (20%) while three samples only were positive for the gene detection by nested RT-PCR (10%) with sensitivity of 100% and specificity of 89% for DFA, this finding is in alignment with several studies that found a great sensitivity for RT-PCR over DFA and viral culture (Maertzdorf *et al.*, 2004). Also, discrepancy in results of RT-PCR may be referred to different genotypes of the virus as it has two main genetic lineages, A and B, with two subtypes for each lineage (A1, A2, B1 and B2). RT-PCR assay may also lack sensitivity due to base pair mismatches in the primer sequences. It is noteworthy that inclusion of appropriate additional primers allows simultaneous assay of multiple pathogens, thus allowing definitive differential diagnosis (Bastien *et al.*, 2003).

In addition to high sensitivity of DFA its rapidity in ruling out negative cases in time which is estimated by maximum two hours should be taken into consideration when evaluating the value of such diagnostic tool. A similar study conducted by Jokela *et al.* (2010) revealed that the agreement between PCR and DFA results was identified by “nearly equal specificity” which goes in accordance with the present study, in spite of the discrepancy in sample size.

In the same context, the present study clarified an agreement between DFA and molecular techniques by (89%) as negative DFA showed evident agreement with PCR negative results except for three samples out of thirty patients (sensitivity ratio 88.9%). This yield of results ensures that DFA is a good negative test. On the other hand, the positivity agreement between two tests was emphasized by a high sensitivity ratio for each of them (100%). Depending on the hypothesis that PCR is the golden standard for diagnosis of hMPV, our study revealed that DFA can be securely used in screening tests for presence of the virus, thus allowing a broad, regular, rapid and easy basis for such tests in future aspects.

Furthermore, Maertzdorf *et al.* (2004) stated that PCR-based tests enabling the detection of a large panel of respiratory viruses from clinical samples are commercially available. However it is not necessary to simultaneously test for all respiratory viruses but rather tests are chosen to detect the prevalent viruses compatible with the clinical picture of the patient. Therefore, a combination of the two methods, DFA as a primary test followed by RT-PCR for DFA-negative samples, may be the best approach for achieving rapid and sensitive detection of hMPV.

## CONCLUSION

Human metapneumo virus infection is a leading cause of respiratory tract infection in infants and the use of DFA provides a primary and easy diagnostic test with high sensitivity and specificity for detection of hMPV in clinical samples. Our study provided a pilot clue for the presence of hMPV among Egyptian children requiring further studies in the same track to provide epidemiologic and clinically relevant data needed to attain a proper vision for the reality condition of hMPV infection and planning for future directions to prevent such infection.



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