

Original Research Article

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Molecular Diagnosis of Human Metapneumovirus Infection among Egyptian Infants with Acute Bronchiolitis

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ABSTRACT

Lower respiratory tract infections are one of the leading causes of morbidity and mortality in children worldwide. Several viruses can cause bronchiolitis. Respiratory syncytial virus (RSV), parainfluenza viruses (PIV), influenza type A, adenovirus and human metapneumovirus (HMPV) are some of the common viruses found in hospital studies. This study aimed to study the molecular diagnosis of HPMV infections by using a real-time, reverse-transcriptase PCR assay and the evaluation of its prevalence among Egyptian infants clinically diagnosed with acute viral bronchiolitis. Also to detect other common respiratory viruses and determine the presence of coinfections of HPMV and other respiratory viruses, we tested the presence of common respiratory tract viruses by using immunofluorescence respiratory screen test in 50 Egyptian infants diagnosed with acute bronchitis and also screened the tested samples for the presence of HMPV by using real-time RT PCR. Our results revealed that 16% of the cases were positive for HMPV, 76% were positive for the other tested common respiratory tract viruses and 12% showed coinfection.

Keywords

Human
Metapneumovirus,
Bronchiolitis, RT
PCR, Infants.

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Introduction

Lower respiratory tract infections are one of the leading causes of morbidity and mortality in children worldwide (Hustedt and Vazquez, 2010). Acute bronchiolitis is defined as an acute inflammation of the bronchiolar airways, which may cause airway obstruction and respiratory distress via bronchiolar wall oedema, spasm and mucous production within the bronchiolar lumen (Sachdeva and Dutta, 2012). Several viruses can cause bronchiolitis.

Respiratory syncytial virus (RSV), parainfluenza viruses (PIV), influenza type A and adenovirus are some of the common viruses found in hospital studies (Debiaggi *et al.*, 2012). Technological advances in the field of molecular biology have allowed virologists to detect many previously undetected viral pathogens (Hustedt and Vazquez, 2010), one of these pathogens was the human metapneumovirus (HMPV) which is a

respiratory pathogen identified in The Netherlands in 2001, and was thought to cause upper and lower respiratory tract infections in children (Van den Hoogen *et al.*, 2001).

It is an enveloped, non segmented, negative-sense RNA virus classified in the subfamily Pneumovirinae of the family Paramyxoviridae (Falsey *et al.*, 2003).

Indeed, HMPV appears to be an important global cause of clinical manifestations, including upper respiratory tract infections, bronchiolitis, and pneumonia in young children. Its epidemiology in Africa is poorly described and factors that allow its recurrent epidemics in communities are not understood (Williams *et al.*, 2004; Owor *et al.*, 2016).

The use of rapid tests for the diagnosis HMPV and other respiratory tract viral infections allows implementation of appropriate infection control measures, thus reducing nosocomial spread, and is useful for consideration of timely treatment with antiviral agents.

The clinical and financial benefits of the rapid detection of viruses in respiratory specimens have been demonstrated in several studies, indicating a direct correlation between a rapid turnaround time and decreased mortality, a decreased length of stay, overall costs, and better antibiotic stewardship (Macfarlane *et al.*, 2005).

The aim of this study is the molecular diagnosis of HMPV infections by using a real-time, reverse-transcriptase PCR assay and the evaluation of its prevalence among Egyptian infants clinically diagnosed with acute viral bronchiolitis. We also aimed to detect other common respiratory viruses and determine the presence of coinfections of HMPV and other respiratory viruses.

Materials and Methods

Subjects

This study included 50 Egyptian infants attending the Alexandria University Pediatric Hospital (Alexandria, Egypt), and clinically diagnosed with acute viral bronchiolitis during the winter/spring season.

Ethical considerations

Under the guidelines of the committee of ethics of the medical research institute (MRI) of Alexandria University, an informed consent was obtained from the parents of each infant prior to sample collection.

Clinical specimens

Two samples were collected from each infant; the first was a nasopharyngeal aspirate collected by using sterile extractor and screened immediately. The second was a throat swab collected by using the standard plastic-shafted virocult® swabs, separated into aliquots and kept frozen at -70°C for further PCR analysis.

Immunofluorescence respiratory screen test

Nasopharyngeal aspirates were diluted by 2 ml PBS and qualitatively screened for the presence of HMPV immediately after collection by using IMAGENTM Respiratory Screen kit (Oxoid, Hampshire, UK) for the detection of RSV, influenza A and B viruses, Parainfluenza virus 1, 2 and 3 and adenovirus in respiratory specimens according to the manufacturer's instructions. This test contains a pool of monoclonal antibodies each of which has individual specificity for either of the above mentioned viruses. The pooled antibody screening reagent is used in a two-step indirect immunofluorescence staining technique (Parija, 2014).

RNA extraction

Extraction of HMPV RNA was performed using the QIAmp® Viral RNA Mini-Kit (QIAGEN, HILDEN, Germany), according to the QIAmp® Viral RNA Mini spin procedure illustrated in the manufacturer's handbook. Before being processed for RNA extraction, samples were first brought at room temperature, centrifuged for 10 minutes at 15000 xg and the supernatant was used for nucleic acid amplification Real time RT-PCR.

Real-time RT-PCR for detection of HMPV

Real-time RT-PCR assay was performed by using the PrimerDesign™ genesig qPCR Detection Kit (PrimerDesign, Southampton, UK) to molecularly detect HMPV in patients' nasopharyngeal specimens by targeting nucleoprotein gene. Detection was made by the Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, United States).

Statistical analysis

Data were organized and processed by the Statistical Package for the Social Science (SPSS) program 22.0 (SPSS, Chicago, IL, USA), using the Chi square test at a 95% level of confidence. A p-value of < 0.05 was considered statistically significant.

Results and Discussion

The age distribution among the 50 infants included in this study is shown in Figure 1. It was found that the peak age of infection was between 1 to three months, and the infections were more frequent in males (72% of the cases). Samples from the 50 infant patients were collected during the winter/spring season over the period of 3 months as shown in Figure 2, most of the samples were collected in January (46%) and this number

decreases in the spring where only 14% of the cases were collected in March.

The clinical and radiological findings observed among the studied cases were shown in Figure 3 where coughing and wheezing were the most predominant findings observed in 98% and 96% of the cases, respectively.

IMAGEN® immunofluorescence respiratory screen test

This test was used to qualitatively detect the viral antigens of any of the 7 most common respiratory viruses that could infect respiratory epithelia and cause lower respiratory tract infections in young children. In this study 38 (76%) cases were positive either to one or more of those 7 mentioned viruses. The cells with positive results appeared with intracellular apple-green cytoplasmic and/or nuclear fluorescence contrasting against a background of red counterstained material. The negative results, on the other hand, showed cells with no intracellular apple-green fluorescence, only background red counter-staining was visible under fluorescence microscopy (Figure 4).

Real-time RT PCR results for human metapneumovirus

Eight cases (16%) were found to be positive for HMPV nucleic acid by using the PrimerDesign™ genesig qPCR Detection Kit.

It was noticed that 6 of the 8 positive cases for HMPV detected by PCR were also positive by immunofluorescence for at least one of the 7 respiratory viruses detected by the IMAGEN® respiratory screen kit (RSV, influenza A/B, parainfluenza types 1-3 and adenovirus). It was also found that 10(20%) of the studied cases were negative for all the tested viruses.

Table.1 Collective results for both Immunofluorescence and PCR

		Immunofluorescence for RSV, influenza A/B, Parainfluenza 1-3 and Adenovirus				Total	
		Positive		Negative			
		Number	%	Number	%	Number	%
PCR results for HMPV	Positive	6	12	2	4	8	16
	Negative	32	64	10	20	42	84
Total		38	76	12	24	50	100

Table.2 Clinical findings in patients with respiratory viruses other than HMPV, patients with HMPV, and patients negative to any of the tested viruses in the study

	Type of viral infection detected						p-value	
	Respiratory viruses other than HMPV* (n=32)		HPMV with or without coinfection (n=8)		Negative to tested viruses (n=10)		P1	P2
	Number	%	Number	%	number	%		
Cough	32	100	8	100	9	90	-	1
Wheezing	30	93.7	8	100	10	100	1	-
Fever	20	62.5	8	100	8	80	0.079	0.477
Feeding difficulties	18	56.2	6	75	3	30	0.439	0.153
Dyspnea	9	28.1	0	0	0	0	0.162	-
Tachypnea	19	59.3	5	62.5	7	70	0.72	1
Vomiting	17	53.1	5	62.5	6	60	0.209	1
Rhinorrhea	7	21.8	4	50	3	30	0.182	0.63
Hypoxemia	6	18.7	2	25	0	0	0.65	0.183
Grunting	1	3.1	2	25	3	30	0.096	1
Diarrhea	1	3.1	1	12.5	1	10	0.356	1
Otitis media	0	0	0	0	1	10	-	1
Hyper-inflated CXR	31	96.8	7	87.5	9	90	0.364	1

* RSV and/or influenza A/B, parainfluenza types (1-3) or adenovirus

p1 compares HMPV- positive patients with patients positive to respiratory viruses other than HMPV.

p2 compares HMPV- positive patients with patients negative to respiratory viruses in the study.

Figure.1 Age and sex distribution among all the studied patients

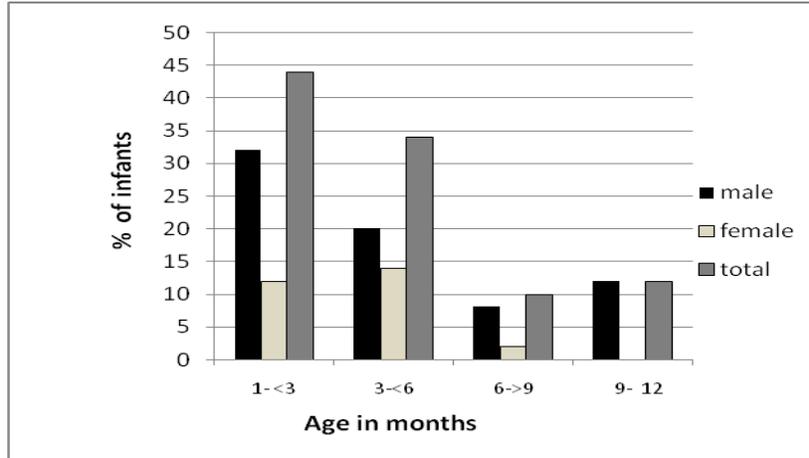


Figure.2 Seasonal distribution bronchiolitis in the studied patients

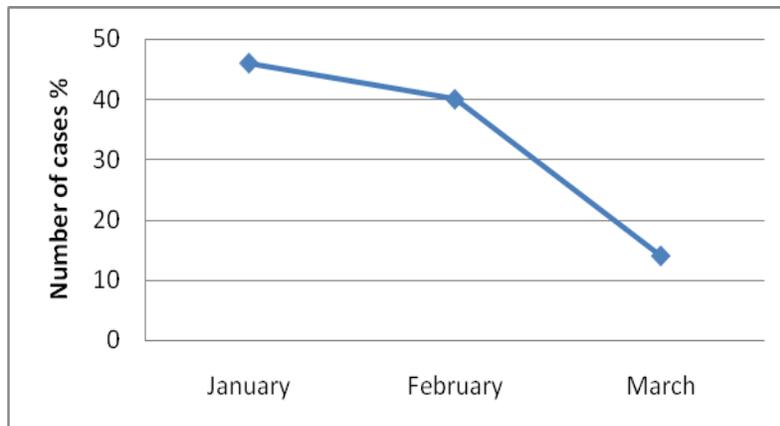


Figure.3 The clinical and radiological findings observed among the studied patients

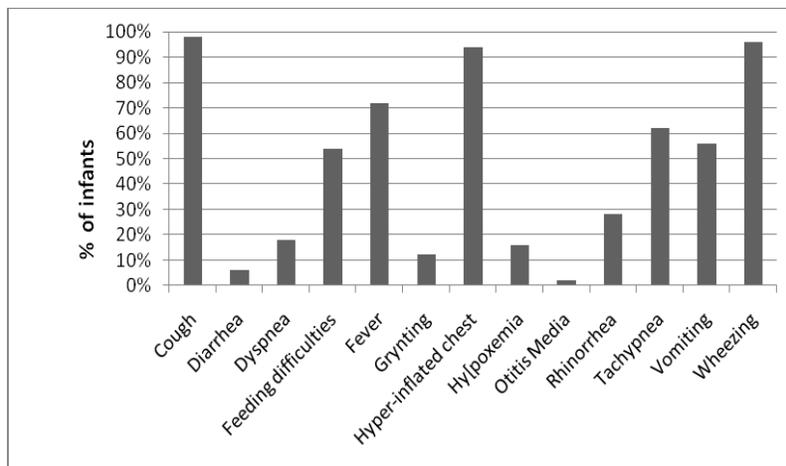
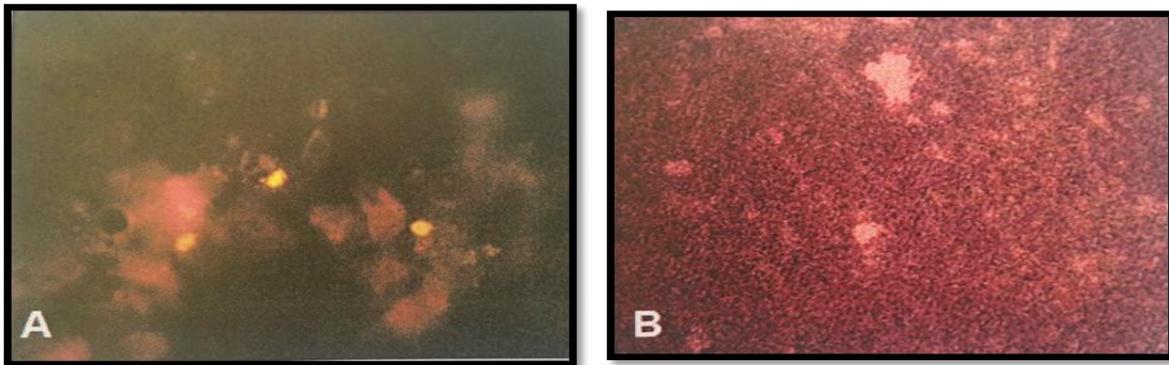


Figure.4 Shows (A) the clinically positive IMAGEN® immunofluorescence slide sample stained and prepared according to the instructions of the kit's manufacturer that appeared to have fluorescence patterns, and (B) A negative diagnosed sample that didn't exhibit this fluorescence



Viral respiratory infections continue to be a worldwide health concern. As the clinical symptoms of patients with acute respiratory tract infections do not usually allow a discrimination of viral or bacterial aetiology, rapid and reliable diagnostic tools are required for better antibiotic stewardship and for the implementation of appropriate infection control measures (Zaas *et al.*, 2014)

According to the Official Journal of the American Academy of Pediatrics, more than one third of children develop bronchiolitis during the first two years of life (Ralston *et al.*, 2014). Another study reported that in infants up to 3 months old, RSV was by far the most common pathogen (58.1%) (Richter *et al.*, 2016). In the present study all the tested patients were reported to be less than one year of age. The peak age of infection was found to be between one month and less than three months of age (44%), whereas only 22% of the patients were in the age of 6 months or above (Figure 1). Similar to our findings, Bradley *et al.*, suggested that young age is a significant risk factor for acute bronchiolitis in children (Bradley *et al.*, 2005), also Owor *et al.*, reported that 73% of the cases diagnosed with HMPV were less than one year of age (Owor *et al.*, 2016). A study of risk factors associated with bronchiolitis in children less than 2 years of age reported that gender, unlike age, didn't show statistically significant association with bronchiolitis (Ruiz-Charles *et al.*, 2001).

However, according to another study the male gender is associated with an increased risk of having a bronchiolitis episode and/ or longer duration (Flaherman *et al.*, 2012). Similarly, in our study the majority of the studied patients (72%) diagnosed with acute bronchiolitis were found to be males.

Bronchiolitis is a seasonal illness having peak prevalence in winter months and early spring (November to March), when such infectious viruses are widespread in the community (Baumer, 2007). In our study, 46%, 40%, and 14% of the cases were found in January, February and March respectively. Similarly Owor *et al.*, found that the cases were predominant in the months of November through April (Owor *et al.*, 2016). In other parts of the world, seasonality in HMPV prevalence has been previously reported (Bastien *et al.*, 2003; Mullins, 2004; Ahmed *et al.*, 2012).

In the present study, a qualitative indirect immunofluorescence screening assay was used to test the presence of seven common respiratory viruses other than HMPV (RSV, influenza virus A and B, parainfluenza virus types 1-3 and adenovirus). Seventy six % of the cases were reported to be positive to at least one or more of these viruses. Another screening study also made by using antigen specific immunofluorescence assay (IMAGEN® respiratory screening test) reported that 60.6% of the tested samples were positive to any of the

respiratory tract viruses (Kleines *et al.*, 2007). The HMPV was detected in 16% of our studies cases. Similarly, Owor *et al.*, showed that this virus was detected in 4.8% of the cases clinically diagnosed with pneumonia (Owor *et al.*, 2016). Other studies also reported that HMPV has been estimated to account for 3% to 19% of bronchiolitis cases (Xepapadaki *et al.*, 2004; Kahn, 2006).

We also studied the clinical impact of HMPV coinfection with other common respiratory tract viruses, and we found that 6 (12%) of the 8 (16%) cases that were found to be positive for HMPV were also positive for at least one of the other seven studied respiratory viruses. In another study, mixed infections were observed in approximately 20% of all the tested samples (Richter *et al.*, 2016). It has been shown that viral coinfection was significantly associated with longer duration of illness symptoms, also severe clinical phenotypes were more prevalent in coinfection patients, especially in RSV, this may increase the severity of RSV associated disease in children (Cho *et al.*, 2013; Wu *et al.*, 2015).

In conclusion the present study revealed that HMPV is an important seasonal contributor to respiratory diseases in Egyptian infants and it is a common cause of acute bronchiolitis that should be considered in etiological diagnosis. It could be found as the sole pathogen or may be present associated with other common respiratory viruses. Future advances in the molecular and diagnostic techniques would help in identifying newly emerging respiratory viruses that were unidentified previously.

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