Viral Infections in Egyptian Hospitalized Children With Acute Respiratory Tract Infections

Niveen M Gad1*, Doaa Refaay2, Naglaa M Gad3 and Zakia A Z Mohamed4

1Microbiology and Immunology Department, Faculty of Medicine, Banha University, Egypt
2Pediatric Department, Faculty of Medicine, Banha University, Egypt
3Clinical Pathology Department, Faculty of Medicine, Banha University, Egypt
4Clinical Pathology Department, Faculty of Medicine (for girls), Al Azhar University, Cairo, Egypt

*Corresponding author: Niveen M Gad, Microbiology and Immunology Department, Faculty of Medicine, Banha University, Egypt, Tel: +201001383106; E-mail: niveengad@yahoo.com

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Abstract

Acute respiratory tract infections (ARTIs) are associated with significant morbidity and mortality worldwide, especially in developing countries. Children under the age of 5 years old are mostly affected. Both viral and bacterial causes are implicated, but viral etiologies are more difficult to diagnose. Viral infections can occur either single or mixed, but the significance and association of polyviral agents with the severity of cases are poorly understood. More studies are critical for understanding this role, improving diagnosis and treatment.

Objective: To identify the rule of five chosen viral pathogens in ARTIs in children below 5 years old and the association of polyviral etiology with the severity of the disease.

Methods: Nasopharyngeal swabs were taken from 120 children who had symptoms and signs of ARTIs, attending the outpatient clinic and admitted in pediatric department or pediatric intensive care unit, Banha university hospital. All children were subjected to full history taking, analysis by multiplex PCR for five viruses (Rhinovirus, Respiratory syncytial virus, Human metapneumovirus, Adenovirus, and Human boca virus).

Results: 54 viruses were identified by multiplex PCR from 41 children (34.2%), 75.6% of them had a single viral infection, (17% and 7.4%) were co-infected with two and three viruses respectively. Positive cases were mostly of children admitted in pediatric ward and the majority were infants. URTIs was the most common presentation followed by bronchiolitis then bronchopneumonia. HRV was the most frequently detected as a single and mixed infection (35.2%) followed by RSV (22.2%). Non-significant correlation was found between mixed infection and the severity of infection.

Conclusion: HRV is the most frequently recognized viral pathogen either as single or mixed in children below 5 years old in our patient group. Mixed infection has no correlation with disease severity. And using Multiplex PCR is an ideal tool for investigating mixed infection.

Keywords: ARIs; RSV; hRV; hMPV; ADV; hBoV; Multiplex PCR; Mixed infection

Introduction

Acute respiratory infections (ARIs) are the principle cause of hospitalization and death of infants and young children in developing countries [1]. Viral pathogens are mostly claimed in ARIs, including Influenza virus (Flu), Human parainfluenza virus (HPIV), Respiratory syncytial virus (RSV), Adenovirus (ADV), and Rhinovirus (RV). Moreover, the significance of newly recognized viruses such as Human metapneumovirus (HMPV), Human bocavirus (HBoV), and another two novel closely related Human polyomaviruses; KIV [2] and WUV [3,4], have been more evident.

Viral ARIs can occur as single or mixed microbial infections, but the significance of these polymicrobial infections has to be investigated. Therefore, accurate diagnosis of viral ARTIs has been shown to reduce the misuse of antibiotics and shorten the duration of hospital stay. Multiplex PCR has readily allowed for the simultaneous detection of pathogens in respiratory specimens. However the interpretation of these studies is confused by the differences of the respiratory pathogen included in the panel of analysis, and the patient population being studied [5]. In this study we chose five viruses to be analyzed including RSV, RV, HMPV and HBoV and AdV. Respiratory syncytial virus is RNA virus of the family Paramyxoviridae and the most common cause of bronchiolitis and pneumonia in young children [6]. Human metapneumovirus, is another RNA virus of the same family, subfamily Paramyxoviridae and the second most common cause of lower respiratory tract infections (LRTIs) of young children. It has been identified also as a significant cause of upper respiratory tract infections (URTI) in young children [7]. Rhinovirus, is RNA virus of the family Picornaviridae and it's the most common cause of common cold, but it also can cause LRTIs [8].
Human bocavirus is a recently identified DNA viral agent that belongs to the family *Parvoviridae*. First isolated in 2005 and may persist and be associated with long-term diseases like lung fibrosis and cancer. Several studies have reported the prevalence of Bocavirus worldwide as ranging from 2 to 21.5%, mainly in children younger than 3 years of age in whom the virus has been associated with URTI and LRTI. HBoVs are classified into species 1 through 4; HBoV1 is predominantly found in the respiratory tract [9,10].

Adenovirus, is DNA virus of the family *Adenoviridae* causing acute respiratory infection predominantly of types 1, 2, 5, 6, occasionally 3 and 7 and account for about 10% of viral respiratory infections. The contagiousness of the virus is related to the virus load [11].

Our objectives were to study the etiology and clinical associations of respiratory viruses in children with ARTIs under 5 years old and to investigate the association of polymicrobial infection with clinical severity.

**Methodology**

This study was a cross-sectional study carried in Pediatric and Microbiology and Immunology Departments, Faculty of Medicine, Banha University, in the period from November 2015 to March 2016. The study was conducted on 120 children with acute respiratory infection visiting pediatric clinic or admitted to Pediatric Department, Banha university Hospital. Their age ranged from 2 month to 5 years. All children were subjected to: full history taking from their parents, complete clinical examination, routine complete blood picture, C-Reactive Protein (CRP) analysis and chest X-ray examination. Cases with positive bacterial cultures were excluded.

**Sample Collection**

Nasopharyngeal swabs were collected from the children, immediately preserved in virus transport medium (VTM) [12] and stored at -80°C prior to testing. The study was approved by the local ethics committee of Banha University Hospitals and written consent was taken from each parent participant [13-16].

**Sample Processing**

(1) *Nucleic acids extraction and purification*

Genomic nucleic acid extraction was performed using The Thermo Scientific Gene JET Viral DNA and RNA Purification Kit (Thermo Fisher scientific, Australia). The kit utilizes silica based membrane technology in the form of a convenient spin column.

The extracted DNA concentration was detected through measurement by UV spectrophotometer. Readings were taken at wavelength of 260 and 280 nm. Purified viral nucleic acid samples were immediately divided into 2 tubes one of them was stored at -20°C and the other was immediately used for reverse transcription.

(2) *Reverse transcription*

For RNA viruses, cDNA was obtained by using an efficient, rapid and DNA cleaning up reverse transcription system (FastQuant RT kit with gDNAse (TiangenBiotech, China).

Ten microliters of a mixture for cleaning up genomic DNA containing (2 μl of 5x g DNA Buffer, 2 μl of the sample and 6 μl RNase free H2O) was added to 10 μl of master mix for reverse transcription containing (2 μl of 10x Fast RT Mix, 1 μl of RT Enzyme Mix, 2 μl of FQ-RT Primer Mix and 5μl of RNase free H2O) and they were mixed by vortex.

The mixture was incubated for 15 min at 42°C followed by incubation for 3 min at 95°C. The obtained cDNA was stored at -20°C.

(3) *Nucleic acid amplification:*

Two PCR sets were performed one is a multiplex PCR for amplification of the purified genomic DNA of DNA viruses (ADV and HBoV), the other is a multiplex RT-PCR for amplification of cDNA of RNA viruses (HMPV, HRSV and Rhino virus).

| Virus | Target | Primer | Sequence (5’–3’) | PCR product size(bp) |
|-------|--------|--------|------------------|----------------------|
| 1. Adenovirus | VP6 | Hex1deg-(F) | GCCSCARTGGKCWTTACATGCACATC | 300 |
| | | Hex2deg-(R) | CAGCACSCCICGRATGTCAA | |
| 2. HBoV | VP1/2 | Boca-AK-VP-(F) | GGTCTCTGCTTAGAAATAGAG | 500 |
| | | Boca-AK-VP-(R) | CCTGCTGTTAGGTCGTTGTTGATGT | |
| 3. HRSV | Fl subunit of fusion (F) protein-(F) | TTAACCAGCAAAGTGTlTAGA | 243 |
| | Fl subunit of fusion (F) protein-(R) | TTTGTTATAGGCATATCATTG | |
| 4. HMPV | L gene-(F) | CACCCCAAGTCCTCTCTGGAAA | 171 |
| | L gene-(R) | CATGC/CACTATAAAGGGTC | |
| 5. HRV | 5NCR-VP4/VP2-(F) | CTCCGCGCCCTGGAATRGCTAA | 110 |
| | 5NCR-VP4/VP2-(R) | TCACGGYRTTCCSYACCAICC | |

**Table 1:** Primers and amplified product length for PCR and RT-PCR used for detection of the targeted respiratory viruses [13-16].
The reaction was performed in a final volume of 50 μL consisting of 25 μL of Maximum Hot Start Green PCR Master Mix (2x) (Thermo Scientific, Australia), 5 μL of template DNA or the cDNA was added to its corresponding PCR tube, 2.5 μL (1.0 μM) of each primer (forward and reverse) specific for each viral genome in each PCR set (Fermentas, Germany), water (nuclease free) to a final volume of 50 μL. All reagents were prior vortexed, and finally 25 μL of mineral oil were added to the reaction mixture. The primers used in amplifications for PCR or reverse transcription (RT)-PCR for each of the targeted viral agents are described in table 1. The selected primers were targeted to conserved regions of viral genomes.

Reactions were carried out in Thermal Cycler (Biometra, Germany). Amplification cycles of multiplex PCR were: Initial denaturation step at 95°C for 4 min, forty repeated cycles of: denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s followed by final extension step at 72°C for 15 min then hold at 4°C. Amplification cycles of multiplex RT-PCR were: Initial denaturation step at 95°C for 4 min, forty repeated cycles of: denaturation at 95°C for 30 s, annealing at 40°C for 30 s and extension at 72°C for 60 s followed by final extension step at 72°C for 15 min then hold at 4°C.

10 μL of each amplified DNA and 100 bp ladder (molecular weight marker) (Fermentans, Germany) were separated on 1.5% agarose gel containing 0.3 μg/ml of ethidium bromide. The bands were visualized using UV transilluminator (Biometra, Germany) (254 nm).

Result

They were 66 males (55%) and 54 females (45%) and their age ranged between (2 months-5 years) old with mean of 1.45. Most of them were under empirical antibiotic treatment. Nasopharyngeal swabs were obtained from all cases and subjected to multiplex PCR for five viruses (RV, RSV, HMPV, ADV and HBoV).

| Table 2: Demographic data and presentation of 41 cases with positive PCR result |
|-----------------|--------------|
| **Source** | **%** |
| Outpatient clinic | 29 (29.2) |
| Ward | 46 (46.3) |
| PICU | 10 (10.6) |

| Table 3: Distribution of respiratory virus infections among 41 positive cases. |
|-----------------|--------------|
| **Respiratory virus** | **Positive Cases** |
| RV | 11 |
| RSV | 6 |
| HMPV | 7 |
| ADV | 5 |
| HBoV | 2 |
| RV&RSV | 11 |
| RV&ADV | 2 |
| RV&HMPV | 4 |
| ADV&RSV | 1 |
| RV&ADV&RSV | 6 |
| RV&ADV&HMPV | 2 |
| Total | 54 |

Discussion

This study was conducted to identify the viral etiologies of ARIs in children younger than five years old in relation to five viruses (RV, RSV, hMPV, ADV and HBoV), and the correlation of polymicrobial infection to severity of clinical outcome. Viral etiology was confirmed by PCR in 41 cases (34.2%) of 120 children; single virus was retrieved from 25.8% of them and 8.3% were coinfected with 2 or 3 viruses. This is less than other studies in which single viral etiology was isolated from 36-80% of studied groups, but it's near or less than other studies in which mixed viral etiologies were isolated from 6-40% of patients.
A study of Cuti and his team mentioned that coinfection is reportedly related to the time of year when circulations of multiple viruses occur, and that there could be likely interplay between climatic, environmental and immunity level that contribute to viral coinfection [5]. The most common age affected by infection in this study was infants ≤ 2 year of age. Even though it was non-significant but this observation was supported by others [17,19]. They suggested that it may be attributed to immature immune system and parents anxiety seeking medical assistance in such young age. On the other hand an interesting finding by Martin and his colleagues found more frequent single infection in infant (birth–5 months) and more frequent mixed infection in (6-23 months) group. They explained that heightened immune response during a primary infection may discourage colonization by a second viral pathogen, leading to lowered prevalence of multiple viruses in young infants [20].

| Respiratory virus | Type of infection | Total No. of isolated viruses (%) |
|-------------------|-------------------|----------------------------------|
|                   | Single infection  | Double infection N (%)           |
|                   | N (%)             | N (%)                            |
| RV                | 11 (20.4)         | 5 (9)                            |
| RSV               | 6 (11)            | 4 (7.4)                          |
| hMPV              | 7 (13)            | 2 (3.7)                          |
| AdV               | 5 (9.3)           | 2 (3.7)                          |
| HBoV              | 2 (3.7)           | 1 (1.9)                          |
|                   |                   | 19 (35.2)                        |

Table 4: Frequency of respiratory viruses according to the type of infections among 41 children.

Double and triple viral co-infection represented (17.1% and 7.3% respectively), which is comparable to other studies that ranged (12-20%) regarding double co-infection and (1.1-3.9%) for triple infection [19,21,22]. RV was the most frequently detected virus in single and mixed viral infection. This agreed with other studies (19,23). Other studies found RSV was the most common as a single virus [1] or as co-infection [5,6]. Al-Ayed and his colleagues mentioned that the most common viruses included in co-infection were RSV and RV [17]. It has been reported the unique characteristic of RSV facilitates infection with a second respiratory virus [20,22]. Al-Ayed and others suggested that hRV in URTI’s could serve as promotion factor which act synergistically to facilitate the pathogenesis of lower respiratory infections such as bronchiolitis [17,20,24].

Liu and his co-authors, suggested the hypothesis that the proportion of the specific pathogen, rather than the pathogen itself, is relevant for co-infections [18]. The discrepancy in proportion of viral agents may be due to differences in pathogen epidemiology, study populations, and/or the time the study was conducted due to seasonal variation. The leading combination in the current study was RV/AdV and RV/RSV, which was comparable to other studies [19,21,22]. Although RSV/ hMPV combination was reported by some studies [17,25,26], this combination was absent in our study and other studies [19,27].

| Isolated viruses | Total n (%) | P |
|------------------|-------------|---|
| HRV n (%)        | 5 (9.3)     | 2 (3.7) | 3 (5.6) | 2 (3.7) | 0 (0) | 12 (22.2) | 0.9 (NS) |
| RSV n (%)        | 7 (13)      | 5 (9.3) | 2 (3.7) | 1 (1.8) | 4 (7.4) | 3 (5.6) | 21 (38.8) |
| hMPV n (%)       | 4 (7.4)     | 3 (5.6) | 2 (3.7) | 1 (1.8) | 0 (0) | 9 (16.7) |
| AdV n (%)        | 2 (3.7)     | 1 (1.8) | 2 (3.7) | 1 (1.8) | 1 (1.8) | 7 (13) |
| HBoV n (%)       | 1 (1.8)     | 1 (1.8) | 1 (1.8) | 0 (0) | 5 (9.3) |

Age/month

Gender

Male

Female

Hospital unit

Clinic

PICU

Ward

Clinical presentations
There is a debate about the significance of the newly identified HBoV as a cause of respiratory infection, which was isolated as a single virus and as a co-infection. Some studies reported that HBoV was isolated as a co-infection [28] and others suggested that the severity of symptoms of HBoV infection is related to viral load rather than the detection of virus [28,29].

| Viruses | URTIs | Bronchitis | Bronchiolitis | Broncho-pneumonia | Pneumonia |
|---------|-------|------------|---------------|-------------------|-----------|
|         | Single | Mixed      | Single        | Mixed n (%)       | Single n (%) |
| HRV     | 10 (18.5) | 1 (1.8)     | 0 (0)        | 1 (1.8)        | 2 (3.7) |
| RSV     | 0 (0)       | 0 (0)       | 2 (3.7)      | 3 (5.6)        | 3 (5.6) |
| hMPV    | 0 (0)       | 0 (0)       | 2 (3.7)      | 3 (5.6)        | 1 (1.8) |
| Adv     | 1 (1.8)     | 1 (1.8)     | 0 (0)        | 1 (1.8)        | 1 (1.8) |
| HBoV    | 2 (3.7)     | 0 (0)       | 0 (0)        | 0 (0)          | 1 (1.8) |
| P       | 0.074 (NS)  | -------     | 0.702 (NS)   | 0.136 (NS)    | 0.154 (NS) |

FET was used

**Table 6**: Correlation of isolated respiratory viruses (mono&co infection) with clinical outcome.

We assumed that mixed infection is more likely to cause more severe infection in the form of clinical outcome, hospitalization or ICU admission, but our study suggested non-significant association which goes in hand with others [4,20,30]. Even though some studies [5,19,21] showed significant association between severity of infection and the detection of mixed viral etiologies. Some studies went further to suggest that single infection resulted in more severe outcome [4,20].

Using PCR as a diagnostic tool is a point for discussion; study of Shafik and coworkers mentioned that viral shedding is mostly at the three days of symptoms; Thus due to the high sensitivity of nucleic acid detection results must be interpreted with caution, particularly if the sample was taken late after symptom onset. So detection of viral co-infection by PCR can detect current and near past infections [1]. Quantitative assays can help resolve this problem. Nevertheless, Alyed and his colleagues mentioned that multiplex RT-PCR is needed for epidemiological and virological data [17].

This study has limitations as it lacks bacterial studies, other viral etiologies and detection of viral loads. Also the short duration of the study made it unable to detect the seasonal variations of viral isolation. In spite of all this, it remains representative of viruses circulating in children under 5 years old in our patient groups. Several common or newly identified respiratory viruses were not assessed in this study, such as Picornaviruses, Coronaviruses, and newly discovered Polyomaviruses, so their contribution to respiratory disease etiology and rates of co-infection in this study remain unknown. These limitation can explain the number of cases which were symptomatic but negative by our PCR study panel.

In conclusion; this study reflected background view of respiratory viral etiology in children younger than 5 years old in Banha, Egypt. HRV was the most frequent viral pathogen either single or mixed infection. Mixed infection has no correlation with disease severity. Additionally, multiplex PCR is a useful tool for detecting mixed infection, but has to be interpreted with caution.

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