Arch Pediatr Infect Dis. 2017 January; 5(1):e36953. doi: 10.5812/pedinfect.36953.

Published online 2016 July 13. **Research Article**

Detection of Respiratory Co-Infections in Children Less Than Five Years With Adenovirus Infection

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Received 2016 February 06; **Revised** 2016 May 15; **Accepted** 2016 June 08.

Abstract

Background: Acute respiratory tract infection is associated with significant morbidity, mortality, and economic loss worldwide. Viral infections seem to be responsible for 80% of the cases. There are several reports on the influence of dual or multiple respiratory viral infections on the severity of disease in childhood.

Objectives: A limited number of studies have been conducted on co-infection of Adenovirus (AdV) with coxsackievirus, human bocavirus (HBoV) and *Mycoplasma pneumoniae* (MP) in Iran. To address this, the present article focuses on both the etiology and epidemiology of multiple microbial respiratory infections (coxsackievirus, HBoV, MP and influenza virus) and their clinical significance in young Iranian children with confirmed AdV infection.

Patients and Methods: Molecular detection of HBoV, coxsackievirus, MP and influenza virus was performed by conventional PCR in 71 respiratory adenovirus-positive samples obtained from children younger than 5 years of age.

Results: Among the 71 adenovirus-positive samples, 6 (8.4%) were co-infected, three were co-infected with MP and three of which were co-infected with influenza A/H3N2. Of the six patients with co-infection, four were male and two were female; three patients were less than 1 year of age and the remaining were 2, 3 and 4 years of age. Moreover, there were two inpatients and four outpatients.

Conclusions: Although several studies have investigated viral respiratory co-infection, no study has evaluated the rate of respiratory co-infections in adenovirus-positive samples from children younger than 5 years. However, this study has filled this gap, the the number of co-infections were too small to draw any definite conclusions. Therefore, large-scale studies using bigger samples are required to understand the clinical significance of polymicrobial acute respiratory infections.

Keywords: Adenovirus, Co-Infection, Coxsackievirus, Bocavirus, Influenza Virus, *Mycoplasma pneumoniae*

1. Background

Acute respiratory tract infection (ARTI) is significantly associated with morbidity, mortality and economic loss, and accounts for approximately 4 million deaths worldwide (1, 2). Respiratory tract infections are caused by a broad range of microorganisms. Viral infections in particular seem to be responsible for 80% of the cases (3, 4). The major causative pathogens are influenza A and B viruses, respiratory syncytial virus (RSV), human parainfluenza virus (HPIV) and human adenovirus (HAdV), which account for 35% to 87% of ARTIs in children (5, 6). In addition, a number of novel human respiratory viruses, including human metapneumovirus (hMPV), coronavirus (CoV), polyomaviruses and human bocavirus (HBoV), are important viral pathogens (7). Several studies have reported that dual or multiple respiratory viral infections may influence the severity of disease in children (2, 8). The incidence of viral respiratory co-infections is often reported between 10% and 20% (9, 10).

Simultaneous occurrence of HAdV and HBoV infection has been reported with high frequency in some studies (11, 12). Adenoviruses are non-enveloped, double-stranded DNA viruses belong to the genus Mastadenovirus within the family *Adenoviridae*. Based on their cell tropism, these viruses are responsible for a variety of clinical diseases such as gastrointestinal, ocular, genitourinary and acute respiratory infections (13, 14). HAdVs can cause a wide range of illnesses, from mild upper respiratory tract infections to serious diseases such as severe pneumonia (15). The detection rate of HAdV is 5% - 7% in young children with respiratory illnesses (16). Unlike RSV, HMPV and influenza virus infections, HAdV infections have been reported mainly in children with viral co-infections (8). The discovery of HBoV in 2005 added a new member to the list of viruses with ability to cause respiratory tract infections (11). HBoV belongs to the genus Bocavirus in the Parvovirinae subfamily of the *Parvoviridae* family which is characterized by a single-stranded DNA genome (17). HBoV infec-

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tions with incidence between 1.5% and 18.3% have been associated with upper and lower acute respiratory infection (ARI) (11). According to the high co-detection rate of HBoV with other viruses, it seems that its replication is partly dependent on other respiratory tract viruses (18).

As both HAdVs and coxsackieviruses cause respiratory tract infections and have a shared receptor, the possibility of respiratory co-infections with these viruses was evaluated in this study. Coxsackieviruses are small non-enveloped RNA viruses that belong to the genus Enterovirus of the *Picornaviridae* family (19). Based on early observations of their pathogenicity in mice, coxsackieviruses are divided into group A and B (20). Group A coxsackieviruses cause herpangina, acute hemorrhagic conjunctivitis, and hand, foot, and mouth disease (HFMD), and group B viruses cause pleurodynia, myocarditis and hepatitis (21). Group A and group B coxsackieviruses can cause respiratory tract disease. The 46-kDa coxsackievirus-adenovirus receptor (CAR) protein mediates the binding and internalization of both group B coxsackieviruses and HAdVs into the cytoplasm (22).

Co-infection with HAdv and atypical pathogens such as *Mycoplasma pneumonia* (MP) is reported to be associated with a higher rate of complications (16). There are a few articles that investigate co-infection with important respiratory viruses such as RSV (23, 24) in Iran. As, there is limited information on co-infection with coxsackievirus, HBoV and *MP. pneumoniae* in Iran. Therefore, this article focuses on both the etiology and epidemiology of multiple microbial respiratory infections (coxsackievirus, HBoV, MP and influenza virus) and their clinical significance in young Iranian children in terms of the incidence rate of HBoV and HAdV co-infections, the use of a shared receptor by coxsackievirus and HAdV, co-infection with HAdV and MP, and the prevalence of influenza virus infections in the community. Meanwhile Influenza virus affect 10-20% of the populations with a different range of outcomes from mild respiratory illness to severe disease or death (14). The prevalence of influenza virus infection will also be studied in this research.

2. Objectives

The aim of this study was to evaluate the prevalence of coxsackievirus, HBoV, MP and influenza virus in respiratory samples positive for HAdV.

3. Patients and Methods

3.1. Sample Collection

In our previous cross-sectional study (unpublished data) that was conducted between December 2013 and August 2014, 71 respiratory samples (throat swabs and nasal washes) positive for HAdV were collected at the National Influenza Center, School of Public Health, Tehran University of Medical Sciences. These samples belong to children younger than 5 years of age. Moreover, questionnaires were distributed to the parents of these patients. From each sample, an aliquot was stored at -70°C for extraction and molecular detection of HBoV, coxsackievirus, MP and influenza virus.

3.2. Nucleic Acid Extraction

RNA and DNA were extracted from 200-μl of samples using the High Pure Viral Nucleic acid kit (Roche, Germany) according to the manufacturer's instructions. Nucleic acid was eluted in 50 μl of supplied elution buffer and stored at -80°C until use.

3.3. cDNA Synthesis

cDNA synthesis was carried out in 30 μl of reaction mixture containing 6 μl of 5 $\times$ RT buffer, 2.5 μl of mixed dNTPs (2.5 μM each), 1 μl of RT-MULV enzyme (Fermentas), 2.5 μl of random hexamers (Fermentas), 0.5 μl of the RNase inhibitor (Fermentas), and 17.5 μl of the RNA template. The mixture was incubated at 22°C for 10 minutes, 42°C for 45 minutes and then 72°C for 10 minutes.

3.4. Primers

Each primer was targeted at a conserved region in the viral genome. NP1 and 5'NTR were selected for detection of HBoV (3) and coxsackievirus respectively. The sequences of the HBoV and coxsackievirus primers are shown in Table 1. For detection of influenza virus, the CDC protocol was used.

3.5. Molecular Detection by PCR

PCR assays were performed for the following viruses. For each set of PCR reactions, negative controls (distilled water) and positive controls were included.

3.6. Conventional PCR for Bocavirus

Bocavirus PCR assay was performed as follows: 10 μl of the nucleic acid extraction was added to 5 μl of 10 x PCR buffer, 2 μl $MgCl_2$, 1 μl dNTPs, 0.4 μl Taq polymerase, 2.5 μl of each primer (Boca-F and Boca-R) and 26.6 distilled water. The protocol used was as follows: pre-denaturation for 3 minutes; 40 cycles at 94°C for 1 minute, 53°C for 1 minute, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. The PCR assay specifically yielded a predicted 354-bp DNA fragment.

Table 1. The Sequences of Primers Used for PCR Amplification of Bocavirus, Coxsackievirus and *Mycoplasma pneumoniae*

Virus	Sequence (5′-3′)	Target Gene	Amplicon Size, bp
Bocavirus		NP1	354
F	GAGCTCTGTAAGTACTATTAG		
R	CTCTGTGTTGACTGAATACAG		
Coxsackievirus		5NTR	500
COF	CGGTACCTTTGTGCGCCTGTTT		
COR1	CGGACACCCAAAGTASTCGGTTC		
COR2	CCGCAGTTRGGATTAGCCGCA		
Mycoplasma pneumoniae		16srRNA	450
F	ACACCATGGGAGCTGGTAAT		
R	CTTCATCGACTTTCAGACCCAAG		

3.7. Hemi-Nested PCR for Coxsackievirus

Ampliqon (Taq DNA polymerase master mix) was used for both rounds of PCR in a 25-μl reaction mixture. For the primary amplification, 12.5 μl Ampliqon master mix, 1 μl (25 pmol) of each primer (COF and COR1), 5.5 μl distilled water and 5 μl of cDNA were used to amplify a PCR product of 600 bp length. The cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. Subsequently, 5 μl of the first-round amplicon was subjected to a second round of amplification using the primers COF and COR2. A 500-bp product was amplified under the conditions mentioned above.

3.8. PCR for Influenza Virus

For detection of influenza virus, the CDC protocol was used (25).

3.9. Conventional PCR for M. pneumoniae

Ampliqon (Taq DNA Polymerase Master Mix) was used for PCR detection in a 50-μl reaction mixture. A 450-bp PCR product was amplified using 25 μl of Ampliqon master mix, 2 μl (25 pmol) of each primer (myc-F and myc-R), 11 μl of distilled water and 10 μl of DNA. The cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 45°C for 30 seconds, and a final extension at 72°C for 10 minutes.

3.10. Electrophoresis

The PCR products were loaded on 1.5% TBE (0.09M Tris, 0.09M borate, and 0.02M EDTA) agarose gels. The gels contained 0.5 μg/mL ethidium bromide for staining. Electrophoresis was carried out for 1 hour at 80 V. Ultraviolet light was used for visualization of the stained DNA samples.

4. Results

4.1. Virus Identification

A total of 71 throat swabs and nasal washes from patients younger than 5 years, which were found to be positive for HAdV by hemi-nested PCR, were tested for the presence of HBoV, coxsackievirus, influenza virus and MP. Among the 71 adenovirus-positive samples, 6 (8.4%) had co-infections, three were co-infected with MP and three were co-infected with influenza A/H3N2.

4.2. Patient Characteristics

The age distribution showed that the majority of patients (83.1%) were under the age of 3 years, and 16.9% of the patients were between 3 and 5 years. Further, 50 (70.5%) and 21 (29.5%) samples were from male and female patients, respectively.

According to the questionnaire, 32 (45.1%) inpatients and 39 (54.9%) outpatients were included in the study. Among the clinical signs and symptoms reported in the affected children, cough (78.8%), fever with temperature above 38°C (60.5%), and sore throat (25.3%) were common findings. Restlessness and myalgia were observed in 11.2% and 8.4% of the patients respectively. The clinical presentations of the six co-infected patients were as follows: five cases of cough, three cases of fever, and three cases of shortness of breath or dyspnea. Among the six co-infected patients, four were male and two were female; further, three patients were less than 1 year of age and the three remaining were 2, 3 and 4 years of age. Moreover, there were two inpatients and four outpatients.

5. Discussion

Co-infection with two or more viruses cannot be distinguished on the basis of clinical presentation (4). In such cases, the treatment goal should be management and control of all the co-infective agents (26). A better understanding of the pathogens involved in pediatric respiratory tract infections and their combined effect on disease severity is required for effective patient care and the development of future strategies for the prevention and treatment of severe ARIs. In this study, we evaluated simultaneous infection of HAdV with a recently identified HBoV and previously known viruses (influenza virus, coxsackievirus and the atypical pathogen MP). Co-infections were identified in 8.4% of the cases, among which 4.2% were co-infected with HAdV and influenza virus and 4.2% with HAdV and MP. None of the HAdV-positive patients were co-infected with HBoV or coxsackievirus. Although in the literature review, there are plenty of studies on viral respiratory co-infections, no study has evaluated the rate of respiratory co-infections in adenovirus-positive samples from children younger than 5 years. In a large study conducted in England, co-infection with influenza A virus and HAdV was associated with increased risk of admission to the general ward (14). In a Swedish study, no correlation was observed between the severity of symptoms in children hospitalized with influenza A infection with or without co-infection (27). Consistent with this finding, no difference was found between the severity of influenza A infection and the presence of respiratory viral co-infections in Spain and Brazil (28, 29). In the present study, the number of influenza A and HAdV co-infections was limited without any severity of the disease.

The clinical significance of respiratory viral co-infections remains controversial, and the major co-infecting viral pathogens vary across studies (20, 30, 31). In the present study, the differences between infections by single or multiple pathogens were not significant in terms of clinical presentation and severity, with the exception of the higher rate of dyspnea observed in the cases of co-infection. Although some studies found that multiple viral infections were associated with more severe fever, a higher rate of hospitalization and more severe disease (20, 32), our findings are in agreement with other studies which have shown that the presence of multiple pathogens was not associated with the severity of the clinical presentation (33, 34).

The clinical presentation of HBoV infection varies widely, and it often involves co-infection with other pathogens. Such characteristics have led to a debate over the role of HBoV as a true pathogen and recently attracted attention globally (35). In the present study, none of the 71 HAdV-positive specimens were positive for HBoV. The highest co-infection rates of HBoV with HAdV reported to date were 37.1% and 69.2% (11, 12). These high rates of co-infection indicate the importance of investigating HAdV in patients diagnosed with HBoV infection.

The differences in the detection level of these viruses between studies could be attributed to differences in geographical location, detection methodologies and sample quality. Moreover, it can be inferred that some viruses have a diverse rate of circulation in different years in the community (20).

So far, only one study has examined dual infection with HAdV and coxsackievirus. In the study conducted by Fujimoto et al., of 100 patients clinically diagnosed with acute exudative tonsillitis highly indicative of HAdV infection, 86 HAdV-positive samples and five (5.8%) HAdV and coxsackievirus dual-infected samples were identified (36). In their study, Fujimoto et al. demonstrated the importance of PCR for the detection of HAdV and coxsackievirus genomes.

The rate of HAdV infection has been reported to be higher in boys (70.5%) than in girls; this may be associated with the smaller diameter of the airways in boys (21). The major symptoms associated with HAdV infection include cough, fever and sore throat. In the present study, the symptoms were almost the same in 6 cases of co-infection and 65 cases of single HAdV infection. The most frequent symptom in both groups was cough. Moreover, there were two inpatients in the co-infection cases and 35 (53.8%) inpatients in the single-infection cases. These results are consistent with studies that have reported similar clinical progression of co-infection and single infections (37). Viral infection enhances bacterial infection by damaging the lung epithelia and increasing the chances of bacterial entry, which is a common side effect of viral respiratory infections (38).

We reported that MP co-infection was present in 4.2% of the cases, which is not rare. In some studies, MP is considered as a cofactor in severe respiratory disease as it dampens the immune response and damages the epithelial cells (16).

As there were a low number of cases of co-infection, it is difficult to draw conclusions from the present results. With the present data, one can only say that the incidence of co-infections is similar between outpatients and inpatients. If other respiratory pathogens had been included in the investigation, the data would have been more comprehensive. A major limitation of the present study is the low number of specimens examined and co-infections detected.

In conclusion, based on the present findings, there is no difference between the outcomes of a single adenovirus infection and co-infection with other mentioned

microbes. Thus, the clinical relevance of co-infection remains difficult to establish. Further studies using larger sample sizes on more viral agents are needed to demonstrate the clinical importance of polymicrobial ARIs.

Acknowledgments

We express our thanks to our colleagues at the Virology Department, School of Public Health, Tehran University of Medical Sciences.

Footnotes

Authors' Contribution: Nazanin Zahra Shafiei-Jandaghi and Somayeh Shatizadeh Malekshahi designed the study and wrote the article; Azadeh Shadab and Maryam Naseri participated in sample collection and viral genome extraction; Somayeh Shatizadeh Malekshahi, Jila Yavarian and Azadeh Shadab carried out the PCR; Nazanin Zahra Shafiei-Jandaghi is the corresponding author; Talat Mokhtari Azad supervised the study; All the authors have read and approved the final manuscript.

Financial Disclosure: None to declare

Funding/Support: This study was funded and supported by Tehran University of Medical Sciences (Grant No. 93.2.27. 25917).

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