

ORIGINAL ARTICLE

Molecular Identification of Species-Specific Adenovirus Causing Respiratory Tract Infections in Pediatric Patients at Benha University Hospital

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ABSTRACT

Key words:

Adenovirus, PCR, respiratory tract infections, pediatrics

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Background: Acute respiratory tract infections (ARTI) are a major threat in children all around the world. Adenovirus is one of the most important pathogens causing respiratory tract infections in children. Adenovirus (AdV) respiratory tract infections is caused mainly by species B, C and E. Accurate and rapid identification of AdV respiratory tract infection is important to avoid unnecessary antibiotic prescription and prevents AdV-related outbreaks. **Objectives:** This study aimed to identify the most prevalent species of adenovirus causing ARTI in pediatric patients hospitalized at Benha University Hospital which were diagnosed by tissue culture and direct immunofluorescence (DIF) test and compare them with polymerase chain reaction (PCR) as a gold standard method. **Methodology:** This study was conducted on 60 pediatric patients suffering from ARTI admitted to Benha University Hospitals. Adenovirus was detected by DIF test and isolated by tissue culture. Molecular identification of species-specific adenovirus was done by PCR. **Results:** Out of 60 cases, 35% were Adenovirus positive by PCR. AdV-B was the most prevalent identified species (76.2%) followed by AdV-C (19.0%) and AdV-E (4.8%). The sensitivity and the specificity of tissue culture and DIF test were 90.5%, 100%, 61.9%, 97.4%, respectively. **Conclusion:** AdV was encountered in one-third of admitted pediatric Egyptian patients with ARTI in Benha University Hospital. PCR was useful for rapid diagnosis of adenovirus infections with higher sensitivity than other methods.

INTRODUCTION

Acute respiratory tract infections (ARTIs) are a persistent and pervasive public health problem in both developed and developing countries, causing nearly four million deaths per year, a rate of more than 60 deaths/100,000 population.¹ ARTIs are a major threat in children responsible for 19% of deaths in children younger than five years of age and 8.2% of all disabilities.²

Many pathogens can cause ARTIs, and viruses have been considered the main pathogens. Viruses are responsible for majority of cases to vary between 40% and 90% globally and ranging from 40% to 50% of infections in infants and children hospitalized with pneumonia in developing countries.³

Adenovirus (AdV) is responsible for approximately 3% to 8% of reported childhood viral respiratory infections. AdV infections can occur endemically throughout the year or in epidemics.⁴

Adenoviruses are non-enveloped double-stranded DNA viruses, classified within the family Adenoviridae, genus Mastadenovirus. Based on their hemagglutination and serum neutralization properties, there are more than 60 recognized human adenovirus types. According to

International Committee on Taxonomy of Viruses (ICTV), 54 types of human adenovirus are grouped into seven subgroups (A to G).⁵

Particular AdV species and serotypes are correlated with specific diseases, epidemiologic settings and demographic risk groups. AdV respiratory tract infection is caused mainly by species B, C and E.⁶ Although respiratory infections caused by AdVs are mostly self-limiting, severe and even fatal infections may occur.⁷

The major types of AdV causing respiratory tract infections change over time in different countries and regions. Therefore, monitoring the types of AdV causing respiratory tract infections in certain regions is important for understanding the prevalence or probably finding a new adenovirus type in certain period.⁸

Early and accurate diagnosis of the causative pathogens in respiratory infections is essential to administer appropriate antiviral or antibacterial therapy, initiate effective infection control measures, and reduce the length of hospital stay.⁹

Laboratory diagnosis can be achieved by viral culture, direct immunofluorescent (DIF) test and PCR assay.¹⁰ Virus isolation in cell cultures is a sensitive method for adenovirus detection, but this method is

costly and time-consuming, taking several days to perform the isolation.¹¹

DIF test is a conventional method that is frequently used in the clinical settings for detection of respiratory viruses including Human adenovirus. DIF test has an advantage of being rapid sensitive test as well as being less labor intensive.¹²

Molecular techniques such as PCR are widely used for the detection and identification of respiratory viruses being helpful in the management of respiratory viral infections. PCR is a relatively quick and sensitive tool, enabling direct laboratory diagnosis in the clinical samples. Depending on the DNA region amplified by PCR, it is possible to distinguish between different species^{13, 14}. This study identified the most prevalent species of adenovirus causing ARTI in pediatric patients which diagnosed by tissue culture and DIF test and compared them with PCR as a gold standard method.

METHODOLOGY

Subjects

This case study was carried out in Medical Microbiology & Immunology Department, Faculty of Medicine, Benha University from January (2019) to April (2020). The study was conducted on 60 pediatric patients suffering from acute respiratory tract infection, admitted to the Inpatient Wards and Pediatric Intensive Care Units of Benha University Hospital. They were 39 males and 21 females. Their mean age was 3.1 years, ranged from 1 month to 10 years. This study was approved by Benha University Ethical Committee and consent was obtained from all parents of children under the study.

Samples

From 60 patients under study, 45 throat swabs, 10 endotracheal aspirates and 5 nasopharyngeal wash were collected and processed for further AdV detection by DIF, tissue culture and PCR. Two throat swabs were collected from 45 patients included in this study. One swab for DIF test and the other for tissue culture and PCR.

Direct immunofluorescence (DIF) test

The principle, procedure and interpretation were performed according to the manufacturer, Spain, Viercell (ACADN).

Tissue culture

Tissue culture was performed on Human Epidermoid carcinoma (HEp-2) cells, kindly obtained from VACSERA Institute at passage No/49. It is a sensitive cell line for isolation of adenovirus. HEp-2 cells were supplied as a monolayer on the surface of the 40 ml plastic culture bottle using Eagle's Minimum Essential Medium (EMEM) containing 5-10% fetal calf serum for serial passages. 50 microliters of specimen was inoculated on to a 24 h old monolayer of HEp-2 cell culture grown over tissue culture bottle after

aspirating out the growth medium. The inoculated culture was kept in a rocker for 30 min. At the end of 30 min, EMEM supplemented with 2 % fetal calf serum was added. The cultures were incubated at 37°C in the CO₂ incubator and observed daily under inverted microscope for the presence of cytopathic effect in the form of rounding, enlarging, clustering and granulation. Continuous checking was done before considering a negative result for up to 2 weeks. The monolayers were fixed with formalin then stained with a 0.1% crystal violet solution and digital photos were taken (Fig. 1, 2). The isolation of adenovirus was confirmed by DIF test.

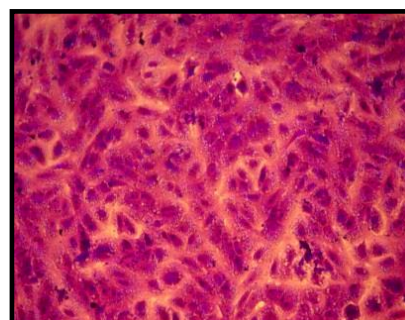


Fig. 1: HEp- 2 cells forming complete sheet of monolayer cells

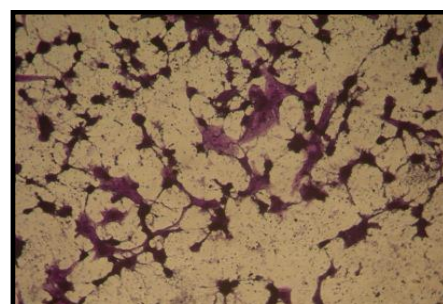


Fig. 2: CPE of AdV on HEp -2 cells

Multiplex polymerase chain reactions (PCR).

Multiplex PCR testing was done for identification of species- specific adenovirus presence. Three primer pairs (Biosearch technologies, USA), specific for each species of adenovirus were used (table 1).

DNA extraction:

Total DNAs of the different adenovirus isolates were extracted by the DNA extraction kit (Thermo Scientific GeneJET Genomic DNA Purification Kit #K0721) according to manufacturer instructions. The extracted DNA was then stored at -20°C until further processing.

DNA amplification:

Amplification was done using My Taq Red PCR Master Mix (2x) (Thermo Scientific, EU Lithuania). The PCR mix contained 25ul of PCR master Mix, 1ul of each forward primer, 1ul of each reverse primer, 5ul of

the template DNA and the amount completed with nuclease free water to reach a final volume of 50ul. Thermal cycler (Biometra, Germany) was used for amplification according to the following program: initial denaturation at 95 °C for 5mins, 45 cycles of denaturation at 94 °C for 30 sec , annealing at 56 °C for 25 sec followed by extension at 72 °C for 15 sec.

DNA detection by agarose gel electrophoresis:

Ten µl of each amplified DNA & 100 bp ladder (molecular weight marker) were separated on 1.5 % agarose gel containing 2µl of ethidium bromide. The bands were visualized using UV transilluminator (312 nm), photographed & analyzed¹⁵ (fig. 3).

Table 1: Sequence of primers used in this study⁴⁰

Species	Primer name	Sequence (5' -3')	Amplicon size (bp)
AdB	AdB1	TSTACCCYTATGAAGATGAAAGC	670-772
	AdB2	GGATAAGCTGTAGTRCTKGGCAT	
Adc	Adc1	TATTCAGCATCACCTCCTTTCC	1988-2000
	Adc2	AAGCTATGTGGTGGTGGGGC	
AdE	AdE1	TCCCTACGATGCAGACAACG	967
	AdE2	AGTGCCATCTATGCTATCTCC	

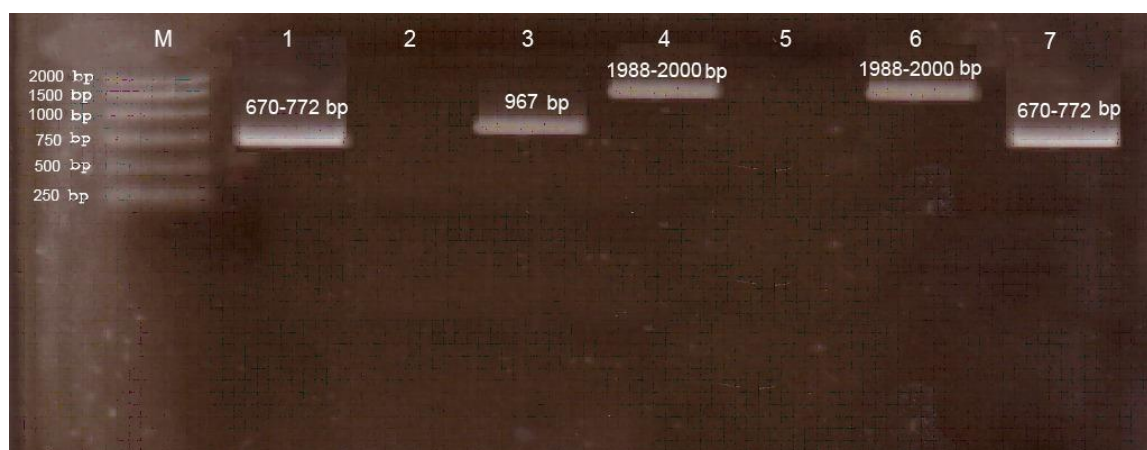


Fig. 3: Agarose gel electrophoresis for the multiplex PCR amplified products of Adenovirus species B, C and E. **Lane M:** represents DNA molecular size marker. **Lanes 1,7:** represent positive Ad-B (670–772 bp). **Lane 4, 6:** represent positive Ad-C (1988–2000 bp). **Lane 3:** represents positive Ad-E (967 bp). **Lanes 2, 5:** represent negative samples.

Statistical analysis:

The collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

RESULTS

Out of 60 cases, 21(35%) cases were adenovirus positive by PCR. DIF test was positive in 14(23.3%) cases and 19(31.7%) cases were tissue culture positive (table 2). Out of 21 cases detected by PCR, 16(76.2%) cases were AdV-B, 4(19.0%) cases were AdV-C and AdV-E was 1(4.8%) case.

Table 2: Adenovirus distribution in 60 studied cases.

	Cases N=60			
	Negative		Positive	
	N	%	N	%
PCR	39	65.0%	21	35.0%
DIF	46	76.7%	14	23.3
Tissue culture	41	68.3%	19	31.7%

Tissue culture and DIF test compared to PCR for diagnosis of AdV pediatric respiratory infections and showed that the sensitivity of tissue culture was 90.5% and the specificity was 100% .Agreement between both methods was very good (k=0.925). The sensitivity of DIF was 61.9%, and the specificity was 97.4%. Agreement between both methods was good (k=0.643) (table 3).

Table 3: The sensitivity and the specificity of DIF and tissue culture in relation to PCR.

		PCR	
		Negative	Positive
DIF test	Negative	38	8
	Positive	1	13
	Sensitivity (%)	61.9	
	Specificity (%)	97.4	
Tissue culture	Negative	39	2
	Positive	0	19
	Sensitivity (%)	90.5	
	Specificity (%)	100%	

61.9% of AdV positive cases were male and 38.1% female. The majority of patients (81%) are < 5 years old. 47.6% of cases were <2 years and 33.3% of cases were aged between (2-5) years with statistically significant difference (P <0.05). The most frequent symptoms among adenovirus infected children were fever (85.7%), rhinorrhea (57.1%), cough (76.2%) and dyspnea (47.6%) (table 4).

Table 4: Comparison of different parameters among negative and positive PCR adenovirus cases.

		Negative PCR		Positive PCR		p
		N=39		N=21		
		No	%	No	%	
Age (years)	mean±SD	3.3	1.1	2.7	0.8	0.346
<2 years	N, %	13	33.3%	10	47.6%	0.527
2-5 years	N, %	18	46.2%	7	33.3%	
≥5 years	N, %	8	20.5%	4	19.0%	
Males	N, %	26	66.7%	13	61.9%	0.712
Females	N, %	13	33.3%	8	38.1%	
Fever	N, %	25	64.1%	18	85.7%	0.076
Rhinorrhea	N, %	22	56.4%	12	57.1%	0.956
Cough	N, %	20	51.3%	16	76.2%	0.060
Dyspnea	N, %	14	35.9%	10	47.6%	0.377
Diarrhea	N, %	10	25.6%	4	19.0%	0.751
Bronchial asthma	N, %	2	5.1%	1	4.8%	0.950
Bronchitis	N, %	2	5.1%	4	19.0%	0.171
Pneumonia	N, %	30	76.9%	9	42.8 %	<0.001
URT	N, %	5	12.8%	7	33.3 %	0.003
Pulmonary disorders	N, %	0	0%	5	23.8%	0.004
Congenital heart disease	N, %	0	0%	2	9.5%	0.119
Prematurity	N, %	0	0%	1	4.8%	0.350
Long hospital stay	N, %	0	0%	1	4.8%	0.350
Genetic disorders	N, %	0	0%	3	14.3%	0.039

DISCUSSION

Adenovirus plays a significant role in pediatric respiratory tract infections, accounting for 2–5 % of the overall respiratory illnesses. ¹⁶ Adenovirus species C, E, and B typically infect the respiratory tract. ¹⁷ Accurate and rapid identification of AdV respiratory tract infection is urgently needed to avoid the overuse of antibiotics, improve the level of diagnosis, even of rarely occurring AdV species and introduction of preventive strategies to avoid the spread of the disease ¹⁵

In the present study, 35% of cases were positive for adenovirus by PCR. This result is similar to Shafiei-Jandaghi et al. ¹⁸ who found that 35.5% of pediatric cases were positive for AdVs in Iran. Calvo et al. ¹⁹ had

the similar observation as AdV was 38% when compared adenovirus infections in hospitalized children with other respiratory viruses.

In contrast, lower prevalence rates of AdV were reported by Qurei, et al. ⁷ who found that 4.2% of AdV isolated from patients of respiratory tract infections in Palestine. Similar findings by Hatem et al. ²⁰ who detected 9.8% among other viral and atypical bacterial etiologies of severe acute respiratory infection in Egyptian patients. It should be mentioned that these studies were not conducted at the same time and in exactly the same ages of children.

It is worth mentioning that such discrepant AdV detection rates can be caused by methodological differences, the number of patients tested, season of sampling, and even a study's duration. Primers and

sensitivity of PCR are possible reasons that have led to different results.²¹

In the current study, AdV-B (76.2 %) and AdV-C(19%) were the most prevalent species within this patient population detected by PCR while AdV-E was (4.8 %). This similar to that found by DOU et al.¹⁵ who detected 72.4% of AdV B, 20% of AdV C and 8% of AdV E detected in respiratory samples of children. Also, Liu et al.²² confirmed that AdV B was 75.3 % of AdV -associated infections.

In Egypt, Metzgar et al.²³ showed a surprisingly high proportion of AdVB (68.1%) infection, compared with general large-scale survey analyses. They explained that AdV B is associated with sever lower respiratory tract infection. The high proportion of AdV B in this population may simply reflect a patient population suffering from relatively severe symptoms.

In contrast, Echavarria et al.²⁴ stated that patients showed mild respiratory infections and 65% of samples belonged to species C, while 11% of species AdV B. In Egypt, over the course of 7 years from 2003 to 2010, Demian et al.²⁵ found that out 60 % of AdV isolates were identified as belonging to AdV-C. Metzgar et al.²⁶ explained the prevalence of AdV-C for it was recovered from upper respiratory tract infections. Whereas AdV-B was more prominent in children with lower respiratory tract infections, in agreement with our findings, from inpatients.

Differences in dominant AdV species was due to factors such as variations in geographic location, time period and sampling methodologies.²⁵

In this study, PCR was compared in relation to tissue culture as diagnostic methods. The sensitivity was 100% and the specificity was 95.1%. Tripathi et al.²⁷ was in accordance with this result, the overall sensitivity of PCR was 100%, specificity and accuracy were 97%. The study confirmed the insensitivity of viral culture for children and indicated that PCR represented a significant improvement over culture. PCR technique was found to be more reliable and useful for rapid diagnosis and was a highly sensitive and specific tool as compared to tissue culture²⁸. DIF test was compared in relation to tissue culture. The sensitivity was 68.4% and the specificity was 97.6 %. This result was in agreement with Echavarría et al.²⁹ who confirmed the sensitivity of adenovirus immunofluorescence assays with respiratory specimens was 60% compared to culture. Puerari et al.³⁰ found that DIF sensitivity was 64.9% and molecular method was more sensible than DIF.

AdV positive samples were isolated mostly from male (61.9%) with no significance relationship between adenoviral infection and gender. This result coincides with other studies in different parts of the world.^{31,32}

In the present study, the vast majority of patients (81%) were < 5 years (1 month- 5 years). 47.6% of cases were <2 years (1 month- 2 years) and 33.3% were (2-5) years with a significant association between them.

In agreement with these results, Stroparo et al.³³ confirmed that majority of patients (89.9%) were ≤ 5 years old. Also, Khalaf, et al.³⁴ found that most patients with AdV infection (85.5%) were younger than 5 years old. Because of the absence of humoral immunity, most of the primary AdV infections happen in the first 5 years of life span.³⁵

The higher frequency of AdVs that cause RTI in young children could be due to one or multiple factors such as: immune system immaturity of young children; physiological population and cultural characteristics and rapid inflammatory responses.³⁶

The most frequent symptoms among positive adenovirus infected children in the present study were fever (85.7%), rhinorrhea (57.1%), cough (76.2%) and dyspnea (47.6%). In accordance with these results, Xie et al.³⁷ declared that fever was one of the most common symptoms after AdV infection (77.1%). Stroporo et al.³⁵ and Moattari et al.³⁸ agreed with our data and reported as fever, cough and dyspnea were the most commonly reported clinical manifestations.

In the current study, AdV positive cases diagnosed clinically as pneumonia (42.8 %) with highly significant value, URT (33.3 %), bronchitis (19.0%) and bronchial asthma (4.8%). In agreement with this result, Wang et al.³⁹ revealed the most common clinical diagnosis was pneumonia and bronchitis (71.1%) in patients infected with AdV-B types with lower proportion of acute upper respiratory infections.

According to this study, underlying co-morbidity associated with AdV respiratory infections in children was 57.1%. Pulmonary disorders, congenital heart disease, prematurity, long hospital stay and genetic disorders were the most frequent illnesses. This proportion is close to a study conducted by Stroporo et al.³³ who defined one or more risk factors were present (64.9%) in patients, mainly represented by prematurity, pulmonary, genetic and cardiac disorders .

CONCLUSION

The present study enabled the construction of a general picture of the common adenovirus species in children in Benha University Hospital. The dominant species in circulation appear to be AdV-B, AdV-C and AdV-E, respectively. Molecular method was found to be useful for rapid diagnosis of adenovirus infections with higher sensitivity than antigen detection and tissue culture.

We recommend identifying adenoviruses at the levels of species, (sero) types, and even strains which is relevant for epidemiological studies, detecting novel AdV incursions and for precise documentation of nosocomial outbreaks. There is a need for continued surveillance in high-risk patients by molecular methods to improve diagnostic flow and efficiency.

Conflicts of interest:

The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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