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ORIGINAL ARTICLES

Introducing two effective techniques for the detection of adenovirus respiratory infection in human sample

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Abstract: *Recently, respiratory viral infections have witnessed substantial diagnostic evolution, with the advent of emergent pathogenic agents and the improvement of new diagnostic approaches. Acute viral respiratory infection (ARVI) commonly causes illness and fatality, particularly in pediatric patients. Adenovirus has the highest prevalence in upper and lower respiratory tract infections (RTIs) amongst respiratory pathogens of importance. The purpose of this research was to introduce two effective techniques for the detection of adenovirus respiratory infection in Human samples, cell culture, and real-time PCR. Samples from patients aged less than ten years were obtained from Baqiyatallah (as) Hospital and cultured on HEK cells after preparation. After observing the cytopathic effects of the virus, a molecular test (Real-time PCR) with the help of specific primers was done on samples taken from the cell culture. Both assays detected viruses in clinical samples, and results of cell culture and real-time PCR confirmed infection with adenovirus in samples. According to our findings, simultaneous use of tests (cell culture and PCR) based test real-time PCR is a somewhat reliable method for detecting adenovirus in clinical specimens.*

Keywords: *Adenovirus, detection, cell culture, real-time PCR*

INTRODUCTION

Adenoviruses were first discovered and characterized in the 1950s by two groups who searched for the etiologic causes of acute respiratory disease (ARD) [1]. Adenoviruses as icosahedral viruses that have no enveloped with an approximate size of 70-90 nm. The viruses were found epidemiologically to be the main causative agent of ARD in recruited militarians [2]. Human adenoviruses are causing merely a minor percentage of ARD in the general population and for almost 5% to 10% of pediatric respiratory disease. Respiratory tract infections by viruses affect children [3]. Adenoviruses are responsible for epidemic outbreaks in soldiers [4]. In addition to respiratory infection, diseases such as conjunctivitis and infantile gastroenteritis caused by adenoviruses can induce fulminant fatal pneumonia, hepatitis, and/or encephalitis in mankind [5]. The respiratory diseases consist of both upper respiratory (pharyngitis and colds) and lower respiratory (bronchiolitis and pneumonia) syndromes [5, 6].

Around 7% of upper RTIs in youngsters aged below five years result from an adenovirus [3]. The respiratory indications are often associated with systemic demonstrations (e.g., generalized malaise, fever, chills, myalgia, and headache). The usual serovars are Ad 1, 2, 5, and 6 (species C or HA

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group III) and, sometimes, Ad3 and Ad7 (species B or HA group I), being indigenous in the majority of populations [7]. Sporadic cases might be indistinguishable from other viral respiratory contagions (e.g., influenza, parainfluenza, and respiratory syncytial virus) [8]. If conjunctivitis comes with the signs and symptoms denoted above, the illness is known as pharyngoconjunctival fever. Ad3 is the adenovirus serovar with the uppermost involvement, but Ad7 and Ad14 isolates among the same HA group have been reported in these patients [9]. Besides, adenoviruses induce lower respiratory tract contagions in youngsters and are a possible cause of nearly 10% of pediatric pneumonia cases [10]. The majority of patients recover from such lower respiratory contagions. However, some pandemics have led to a remarkable fatality, respiratory symptoms duration of almost one week, ocular symptoms for 2–8 weeks, and gastrointestinal symptoms up to 10 days [11]. Adenoviruses are detectable through isolating in nasopharyngeal and conjunctival discharges and throat washes and are visualized via electron microscopic assay of feces in the course of the acute disease phase [12]. Respiratory infections typically represent an elevation in CF antibodies to a common antigen. Virus-related antigens are also detectable in samples clinically through various immunologic methods and PCR [13]. The current research aimed to evaluate the detectability of adenovirus respiratory infections by two techniques, cell culture, and real-time PCR.

MATERIALS AND METHODS

Origin of samples: In this study, samples from patients less than ten years old were received from the Baqiyatallah (as) Hospital and prepared as oral swabs from patients with the common cold and pharyngitis symptoms. In most cases, NP swabs are obtained from elder children and adults, whereas newborns and youngsters are the common sources of NPAs. Since most patients cannot tolerate NP sampling well, other samples were obtained as well. Clinical specimens were transferred to the laboratory on wet ice to preserve viral vitality. These samples were placed in a transport media.

Cell culture: Human embryonic kidney cells (HEK) were cultured in three T25 flasks grown in Dulbecco's altered Eagle medium (DMEM (1X)) complemented with 10% fetal bovine serum (FBS). After inoculation, cell culture tubes underwent incubation at 37 °C and 5% CO2. Samples of patients in the two flasks were inoculated, followed by adding the maintenance medium (DMEM (1X) supplemented with 2% FBS) under the above conditions. Up to a week daily, flasks were examined microscopically to record the viral cytopathic effect (CPE) [14].

Nucleic Acid Purification and Real-Time PCR: A pair of primers were designed for the hexone locus, with forward (5-ACCTGAGTCCGGGTCTGGTGC-3) and reverse (5-GTCAACGGGCACGAAGCGCA-3 primer sequences, as well as a probe sequence of GATGTGACCACCGACCGTAGCCAGCG. Extraction of nucleic acids from respiratory specimens was done by a DNA kit (High Pure Viral Nucleic Acid Kit - Roche) following the manufacturer's instructions. In the next step, a pair of primers was designed for the hexon gene of the adenovirus for specific detection of the virus according to the melt curve. Each reaction had a total volume of 20 µL, including 10 µL of Mastermix PCR probe base, 5 µL of template, 1 µL of primer (0.5 µM), 1 probe (0.5 µM), and 3H2O. The cycling conditions included a primary denaturation step at 95 °C (2 min), followed by 60 cycles (each for 20 sec) at 95 °C, and at 57 and 72 °C for 40 and 30 sec respectively. The annealing temperature was 57°C. A melting curve analysis program was used to identify specific PCR products. After the last cycle, the temperature was increased to 95 °C, then decreased to 75 °C, and slowly elevated to 95 °C at a rate of 0.1 °C per second. Positive and negative controls were added in each set [15, 16].

RESULTS

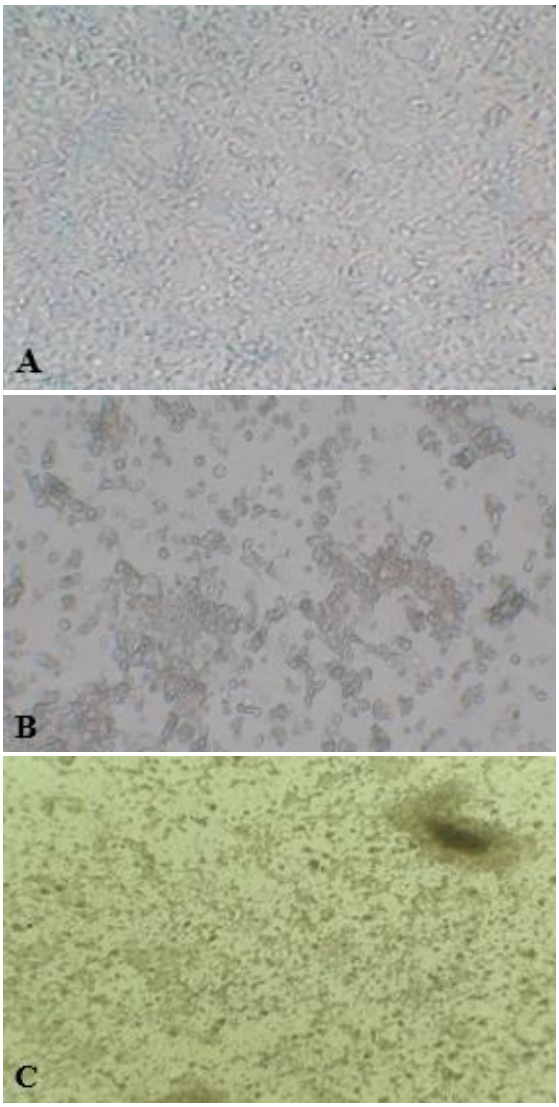
The basis of this study was to show that the involvement of viruses can be an important factor in the upper respiratory system of patients. In this study, adenovirus was studied as one of the causes of conflict in the upper respiratory tract of children.

The samples used in our study were taken in Baqiyatallah (as) Hospital from patients with ages of fewer than ten years who had symptoms such as common cold, pharyngitis, and fever. As permissive cells for adenovirus, HEK cells infected with the virus show typical CPE that resembles grape-like clusters. HEK cells were cultured in three T25 flasks grown in Dulbecco's altered Eagle medium (DMEM (1X)) enriched with 10% FBS and 1% Pen Strep (10000 Unit/ml penicillin (P-4687) + 10000 µg/ml streptomycin (S-1277) Bio-Idea made from SIGMA powder).

When the cell confluence in the flask was approximately 100%, the patient samples were inoculated by the cells in an incubator with CO2 for one hour, after which samples were washed with PBS and added to each flask containing maintenance medium (DMEM (1X) enriched with 2% FBS and 1% Pen Strep).

Flasks were incubated in an incubator at 37 °C and 5% CO2. Flasks were checked daily to observe CPE. On the third day, CPE started formation in the two flasks infected with the virus, which included rounding of cells to clustering. Almost on the seventh day, grape-like clusters, which are typical CPE for adenovirus, were visible (Figure 1).

Figure 1: The cytopathic effects in cells after inoculation. A) Control cells that had not been inoculated with the virus. B & C) Patient samples 1 and 2 in the two inoculated flasks. Cytopathic effect was evaluated daily. The result was observed associated with adenovirus infection including grape-like clusters by separating the cells from the bottom of the flask

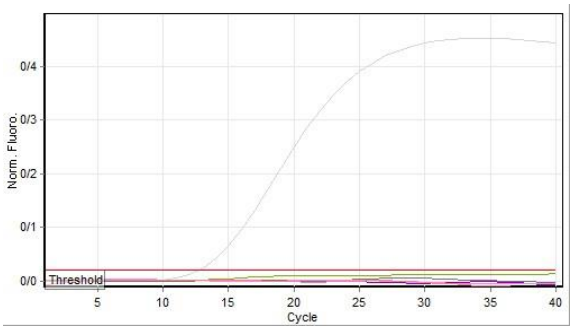


According to the figure of real-time PCR, clinical samples were positive for adenovirus in the form of double. The diagram shows that the amount of virus in sample 1 is greater than that of sample 2 (Figure 2A). Figure 2B shows real-time PCR results and related explanations.

DISCUSSION

Virus-related respiratory infections are prevalent during the year in all age clusters and all populations worldwide. Respiratory viruses can induce moderate ailments, including the common cold, or acute sickness (e.g., pneumonia), which affect both immunocompetent and immunocompromised hosts [17]. Despite the infection of both the upper and lower respiratory tract by every respiratory virus, special syndromes (e.g., croup or laryngotracheobronchitis) happen clinically by particular viruses. Both DNA and RNA viruses are reported to induce respiratory infection [18]. Researchers have described several novel viruses that have relationships with respiratory illnesses [19]. Pandemics of adenovirus respiratory infection are prevalent during winter, spring, and early summer, with protracted (a length of 1–3 years) durations [20]. Almost 80% of upper RTIs occur by viruses etiologically and can exacerbate asthma and acute otitis media [21].

Figure 2. A) Adenovirus molecular test results (real-time PCR) confirming the presence of adenovirus. In this test, a sequence-specific primer pair and a probe were used for the viral hexon sequence. B) Shows real-time PCR results and related explanations. In this test, other viruses were used as a negative control, the results of which are obvious.



No.	Colour	Name	Type	Ct
22		HSV1	Negative Control	
26		NTC	NTC	
28		Adenovirus	Positive Control	12/69
31		HSV2	Negative Control	
37		CMV	Negative Control	
40		BKV	Negative Control	

Adenoviruses are the leading respiratory pathogenic agents and account for 5-8% of all RTIs in newborns and youngsters aged two years [22]. HAdV infections have clinical similarities to those of other respiratory viruses, and it is difficult to decisively detect and swiftly diagnose the virus type, which is not achievable clinically [23]. Due to the persistency and protracted casting of adenovirus, methods of diagnosis should have sufficient sensitivity for detecting small amounts of virus in clinical samples. Several laboratory methods are available to detect adenoviruses, including nested PCR, real-time PCR, PCR, IF, and cell culture, of which molecular and cell culture techniques are sensitive enough to detect the virus [24]. The viral antigen is swiftly detected by IF but is mostly not sensitive to detect some viruses;

hence it is occasionally required to be confirmed by viral culture [25]. Molecular analyses based on polymerase chain reaction (PCR) are gaining ground for diagnosing virus-related respiratory infections and conducting epidemiologic examinations [26]. As a benchmark, viral culture was well-known to test viral respiratory pathogens. Even so, the technique suffers from slowness in general, which usually requires over 14 days to deliver results [27]. Although the number of positive results can be increased by combining these two procedures, existing reports indicate that a considerable number of samples remain negative despite suspicious viral infections both clinically and epidemiologically [28]. During the last years, a growing body of evidence has been presented on the development of molecular biology diagnostic methods for viral infections, which will undoubtedly be the benchmark approaches presently applied in ordinary laboratory assays [29]. Molecular procedures significantly contributed to the fast detection of pathogenic viruses accompanying acute respiratory infections, which allow for rapid implementation of therapeutical actions and preemptive policies to prevent disease dissemination with high transmission ability [30]. Studies in Iran, Kenya, Brazil, China, Australia, and Hong Kong showed that 5-22% of samples were affected by adenoviruses [31].

Given the importance of cell culture and real-time PCR methods in recognition of respiratory adenovirus, cell culture was confirmed by real-time PCR in this study. Their grape-like clusters identified the CPE of the adenovirus in the cell culture. From the results of real-time PCR, it is concluded that the upper respiratory tract symptoms in children of our study might have arisen by adenoviruses. Suppose a large number of patients could be studied epidemiologically on this subject and in case of obtaining statically significant results. In that case, the season of adenovirus infection frequency can be verified in the studied district to demonstrate possible differences in the season frequency of infection.

CONCLUSIONS

This study showed that simultaneous use of tests such as cell culture and PCR-based tests, including the Real-time PCR, are somewhat reliable methods for detecting adenovirus in clinical specimens.

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Conflict of interest:

The authors report no conflicts of interest.

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