Determination of the Genotype - Adenovirus That Causes Multiple Infections

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Abstract

Background: Human Adenovirus (HAdV) is considered one of the important viruses that infect humans and cause multiple infections in the body, it is one of the important causes of conjunctivitis, respiratory and gastrointestinal infections, causing diarrhea. The current study was done to determine the genotype of HAdV in Egypt.

Materials: The study was conducted from November 2021 to November 2022. 120 samples (90 stool samples and 15 conjunctival and 15 respiratory samples) were collected from different regions of Egypt, Information related to age, sex and region were recorded. The HAdV was detected serological by ILFST test. The positive samples were cultured and HAdV isolated on Vero cell lines. HAdV was collected from infected cells and then identified using the multiplex PCR technique. The genotype HAdV was determined through bioinformatics.

Results: Through this study, ILFST test, HAdV was detected in 20 (22.22%) out of 90 stool samples, while the conjunctival and respiratory samples was negative. 12 out of 20 samples were cultured on Vero cells. The cytopathic effect CPE was observed with 6 out of 12 samples. Three samples were identified as adenovirus using the multiplex PCR, and confirmed by agarose with expected 350 bp. The nucleotides sequences of 3 isolates were alignmented with HAdV isolates recorded in gene bank with 96-98 %. The three HAdV isolates appeared that belong to genotype F-40 by phylogentic tree. Interestingly, the Human adenovirus genotype F-40 was the most common genotype in Egypt. our work revealed the infection rate, virus isolates, genetic diversity, seasonal distribution, of HAdV infections in samples in Qalyubia, Cairo and Alexandria. Through this study and previous studies that converge with it in some results in Egypt and some countries of the world, enteric adenovirus genotype F especially the serotype 40, play a key role in gastroenteritis especially for those under five years old in Egypt.

Keywords: HAdV, ILFST, multiplex PCR, Vero cell lines

Introduction

Human Adenovirus (HAdV) is a naked medium-sized icosahedral particle, 70–90 nm in diameter. It contains a strand that consists of a double-stranded DNA molecule. It belongs to the genus Mustadenovirus in the family Adenoviridae(1). The genus Mustadenovius is divided into seven species, A through G, based on biological, immunological, and biochemical characteristics (2). To date, over 70 genotypes are known. Human Adenoviruses infect multiple sites of the human body with different genotypes (3) Among these species, the enteric genotypes HAdV-12, AdV-18, AdV-31 (4-5) **.** It is reported that because no effective treatment for viral gastroenteritis other than a rotavirus vaccine is available, (6) local and local epidemiological data on adenovirus infection are needed to help health professionals and authorities develop and implement appropriate vaccination and control measures. essential to implement. Also, HAdV infection causes mild to moderate in the body (7). Immunocompromised children, serious illness can occur in young children with a weakened immune system Patients and those with chronic underlying diseases This can result in significant morbidity and mortality (8). Also, adenovirus can infect Respiratory tract infections (RTIs) are one of the leading causes of death in children, especially in developing countries (9-10). Also, adenovirus can infect. Human keratoconjunctivitis is 1, 4, 5, 6, 8, 19a, 37, 53, 54 and 56 (11) The current study aims to determine the genotype of adenovirus among infected patients in different regions of Egypt.

Material and Methods

Sample collection:

Ninety fecal samples of children were taken from suspected cases showing symptoms of diarrhea, weakness and fever, also tearing, redness and eyelid swelling collected from Qalyubia, Cairo and Alexandria governorates. Records of complete patients' history, such as age, genus, date of onset of symptoms, and type of symptoms were noted. The samples were kept in pure environments and at an optimum temperature to preserve the vitality of the sample and prevent any contamination until the examination was conducted (12)**.**

Preparation of stool Samples:

A total of 90 stool samples were collected from the three governorates Qalyubia, Alexandria and Cairo. ninety watery-diarrheal samples negative for Rotavirus were selected, and diarrheal samples with blood were excluded from this study. After exclusion of rotavirus Ag, the samples were

processed, and the stool supernatants were stored at (-80 °C).

Preparation of conjunctiva and respiratory swabs samples:

15 conjunctival and 15 respiratory swabs (men and women) were collected from patients with suspected viral conjunctivitis and respiratory infection based on clinical features (at least one of the complaints below: redness, rhinorrhea, pain, acute bronchitis, fever, eyelid swelling and tearing). Take one specimens were retrieved from each patient with sterile Dacron swabs. The swab was employed for IC adenovirus test.

Detection and isolation of Adenovirus:

Human Adenovirus was detected by ILFST test and then by vero cell culture approach and then confirmed by PCR. First, ILFST test directly by added to the sample wells of the kit according to the kit manufacturer's instructions (**Hangzhou Biotest Biotech**). Results were read within 15 minutes. and then by inoculating the stool superannuates into the Vero cell lines. After that, molecular detection was performed to confirm the presence of the virus and to know its genotype.

ILFST test:

ILFST test were performed directly and 150 μl samples were added to wells of the kit according to the kit manufacturer's instructions (**Hangzhou Biotest Biotech**). Results were read within 15 minutes. There are three lines that can identify a sample as positive or negative. If one line appears on the control position line, it means the sample is negative, if two lines appear on the control position line and T1 line, it means it is positive. After a positive stool sample appears for adenovirus (13).

Isolation and propagation of HAdV on Cell culture:

Samples that gave a positive result in the ILFST test were cultured on Vero cells from the VACSERA Institute. This is a sensitive cell line for adenovirus isolation. vero cell were provided as monolayers on the surface of 40 ml plastic culture flasks and serially subculture using Dulbecco's Modified Eagle Medium (DMEM) containing 5–10 μl bovine serum. After aspirating the growth medium, 50 microliters of stool solution were inoculated onto 24-hour old monolayers of vero cell cultures grown on cell culture flasks. The inoculated culture was kept on a shaker for 30 minutes. After 30 minutes, 2 μl of DMEM supplemented with bovine serum was added. Cultures were incubated at 37° C in a CO_2 incubator and observed daily under an inverted microscope for the presence of cytopathic effects in the form of rounding, spreading, clustering and granulation. Monolayers were fixed in formalin, stained with 0.1% crystal violet solution, and digitally photographed (14).

Molecular detection of genotype-adenovirus

Extraction of DNA: DNA was extracted from the cell culture using a QIAamp DNA Mini kit (**Qiagen, Hilden, Germany**), as directed by the manufacturer. To summaries, DNA was extracted from 140 μL of each sample to yield 60 μL of DNA solution. The quality of the DNA was determined using a Nanodrop Spectrophotometer (A260/280 ratio). The extracted DNA was frozen at -20 °C until Multiplex PCR.

Proceeding of Multiplex Polymerase chaic reaction (Multiplex PCR).

DNA was purified from the PCR products using MinElute PCR purification kit (**Qiagen**) according to the manufacturer's recommendations using hexon gene specific primers for AdV detection, (15) and penton gene specific primers for genotyping (16) The total reaction volume of 50 mL, which contained 20 pmol/mL of each primer, 2.5 mM of dNTP, 1.25 U of GXL DNA polymerase (**Takara, Shiga, Japan**), and 5 mL of the DNA template. Thermal cycling was performed for 30 cycles; each cycle consisted of these following steps: denaturation at 98°C for 30 sec., annealing at 55°C for 30 sec., and elongation at 68°C for 2 min, followed by a final extension step at 68°C for 7 min. The PCR products were separated on a 3z agarose gel for 50 min at 100 V. DNAwas purified from the PCR products using MinElute PCR purification kit (**Qiagen**) according to the manufacturer's recommendations. The nucleotide sequences were determined by using the Big Dye Terminator cycle sequencing kit v3.1 (**Applied Biosytems, Foster City, CA, USA**) and an ABI 3130XL genetic analyzer.

Table (1): Primer sequences for detection of genotyping of enteric adenoviruse

Gene primers	Name	Sequence	Fragment size
Hexon PCR	H1	5′-TTGACATCCGCGGCGTGCTG-3′	308 bp
	Ad4	5′-TATTCTGAGACCAGTTAGTT-3′	
	Ad41	5′-CTGCAGTCCAGGTTTGGCCA-3′	
Penton PCR	Ad-penUF	5- CARAAYGAYCACAGCAACTT-3	484 bp
	Ad-penUR	5-GCRGGMACGTTTTCACTRACGGT-3	

Gel Electrophoresis:

The PCR products were separated on 2% agarose gels for 50 min.at 100 Volt. Agarose 2% was prepared in 100 ml of 1X TBE buffer (W/V) and heated in water bath and added 5μl of ethidium bromide (17). 5μl from one Kb DNA ladder and 10

µl of PCR products were loaded in each well. The DNA fragments were viewed under UV transilluminator and immerged by digital camera.

Sequencing:

For the extraction of specific product of approx. 350 bp size, GenElute™ Gel Extraction Kit **(Sigma-Aldrich, USA)** was used. Extraction was carried out according to manufacturer's protocol. Sequencing was performed in a single direction using the internal forward primer (AdHex F2) and the BigDye®Terminator v3.1 Cycle Sequencing Kit **(Life Technologies, USA)** on an ABI 3500 sequencer **(Life Technologies, USA).**

Nucleotide sequence analysis:

Genotypes were identified using Genbank BLAST tool from NCBI, with identity score ≥97 %. The hexon gene sequences of another 33 Adenoviruses (different types obtained from GenBank) were included as reference strains for phylogenetic analysis. These strains were isolated from different parts of the world during different time periods.

Phylogenetic analysis was done by neighbor-joining method with 1000 repetitions (bootstrap value: 1000) by Mega 5.2 software. Analysis of recombination in hyper variable region was performed using the software Simplot 3.5.1.

Results

Recorded Information about the patient, such as age, genus, date of onset of symptoms, and type of symptoms were noted, confirmed and determined the type of virus infection, (**Table, 2 and Figure, 1).**

Table (2). Total number of collected samples from suspected HAdV infection.

Gender	Collected samples (N=90)	Percentage (%)
Male	52	57.7
Female	38`	42.3

samples collected from suspected
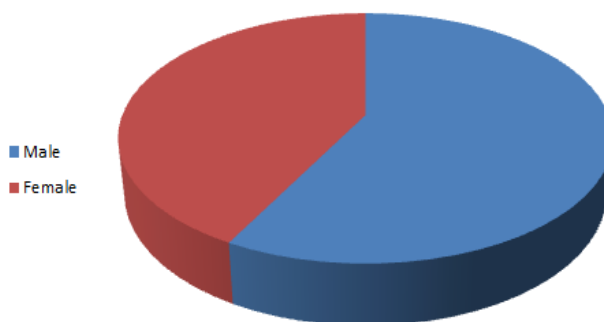

■ Male
■ Female

Fig (1): Histogram showing the total number of samples collected from suspected HAdV infection1. ILFST test.

Serological Detection of HAdV

According to the ILFST test, (20) positive samples were detected out of a total of 90 (22.2%) samples of patients showing symptoms of Human adenovirus infection **(Figure, 2).**
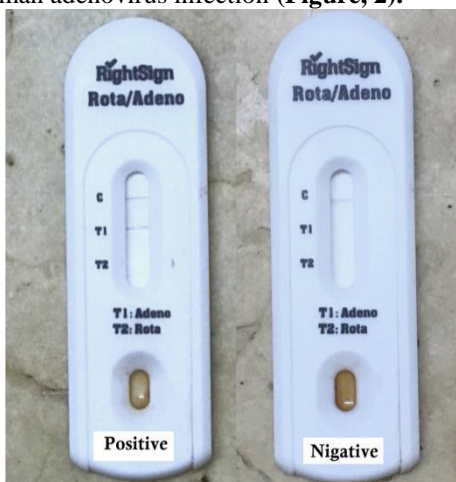

Fig (2). The results are interpreted as follows, two colored lines on the test stick (C and T1) are positive for adenovirus (HAdV) antigen one colored line is negative for adenovirus (HAdV) antigen. ILFST test.

Detection rate according to Gender:

Watery diarrheal samples were collected from male (52) (57.7%) and female (38) (42.2%) out of 90 patients. According to the ILFST test, among these a total of (n=13/52) 25% while (n=7/38) 18% females with acute diarrhea were tested positive for adenovirus. There was a significant difference observed in the positivity rate regarding the gender. While the conjunctival and respiratory samples was negative (**Table 3, Figure 3**).

Table (3): The Total number of samples according to the ILFST test, collected from people infected with adenovirus.

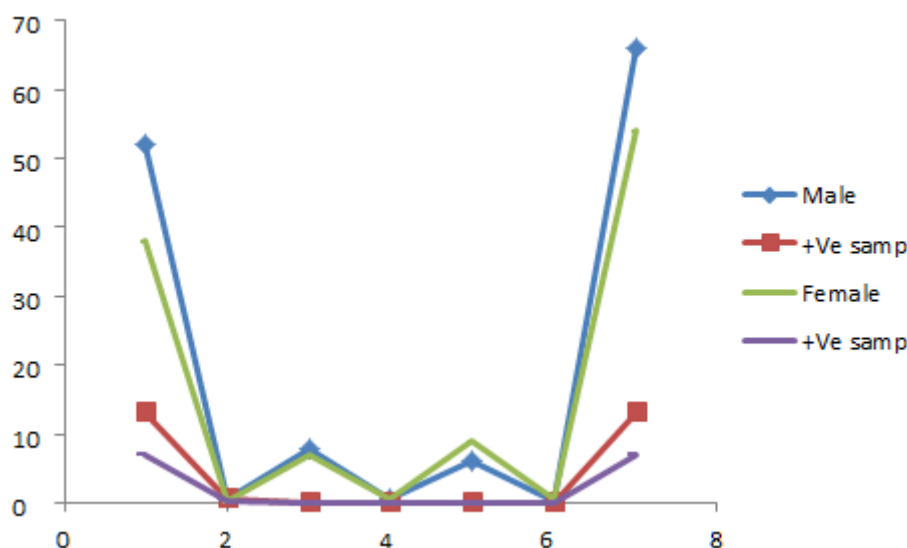| Samples	Male		Female	
	Total samp.	+Ve samp.	Total samp.	+Ve samp.
Total stool samples	**52**	**13**	**38**	**7**
Rate % of total stool samples	**57.7%**	**65%**	**42.3%**	**35%**
Total Conj. Samples	**8**	**0**	**7**	**0**
Rate % of total Conj. Samples	**100%**	**0**	**100%**	**0**
Total Resp. samples	**6**	**0**	**9**	**0**
Rate % of total Resp. samples	**100%**	**0**	**100%**	**0**
Total samples		**120**		

Fig (3): Histogram showing of positive samples according to the ILFST test, collected from people infected with HAdV. regarding Gender group.

Detection rate by age group:

In this study, the studied population was divided into three age groups. According to the ILFST test, the highest positivity rate for HAdV was noted that (10 sample) of HAdV-infected children were 12-20 months (male 6 and female 4). While the lowest rate positivity rate for HAdV was noted in the results of children infected under 12 months of age (4 samples /male 3 and female 1). While the number of positive samples in the age group from 24-60 months was (6 samples / 4 males and 2 females) (**Table 4, Figure 4**).

Table (4): Age groups at risk of HAdV infection according to the ILFST test.

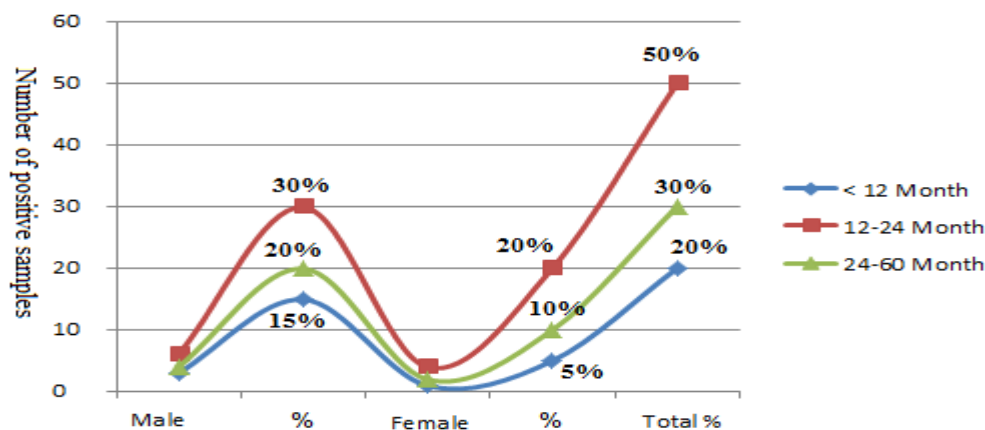| Variable		+Ve Stool samp.		+Ve Stool samp.		% of total
		Male (n=20)	%	Female (n=20)	%	
Age years	**< 12 month**	**3**	**15%**	**1/20**	**5%**	**20%**
	12-24 month	**6**	**30%**	**4/20**	**20%**	**50%**
	24-60 month	**4**	**20%**	**2/20**	**10%**	**30%**

Fig (4): Histogram Age groups most at risk of HAdV infection, according to the ILFST test.

AdV isolates detection on cell lines culture:

Twelve samples, which gave positive for serological ILFST test   cultured on Vero Cell for three days. Samples were followed up for a week. The results were showed 6 gastrointestinal (7 , 8, 9, 10, 11, 12) showed cytopathic effects (CPE**) while 6 samples 3 collected from** conjunctivitis , (1 ,2, 3) and 3 samples collected from respiratory (4 , 5 , 6)

no showed cytopathic effects (CPE) a showing in the **(Table 5 , Figure 5, 6 and 7) .**The results of cytopathic were followed up on the first, second, and third days, and the results appeared as follows: After 24 hours ,was showed a positive cytopathic effect on 3 samples. After 48 hours, was showed a positive cytopathic effect on 5 samples. After 72 hours, was showed a positive cytopathic effect on 6 samples.
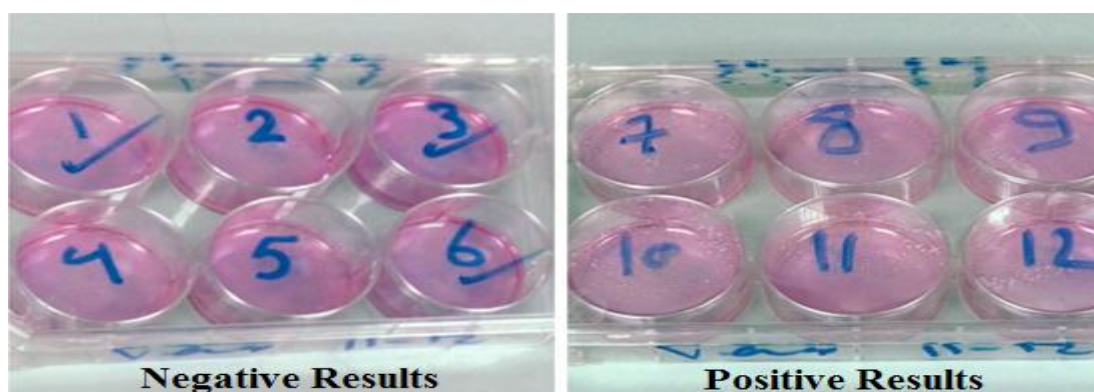

Fig (5): Samples cultured on Vero cell lines cultivated with 12 samples collected from conjunctivitis, (1 ,2, 3); respiratory (4, 5, 6) no showed cytopathic effects (CPE) and gastrointestinal (7, 8, 9, 10, 11, 12) showed cytopathic effects (CPE).

Table (5): Effect of adenovirus-induced cytopathic on type of cell culture Vero cells .

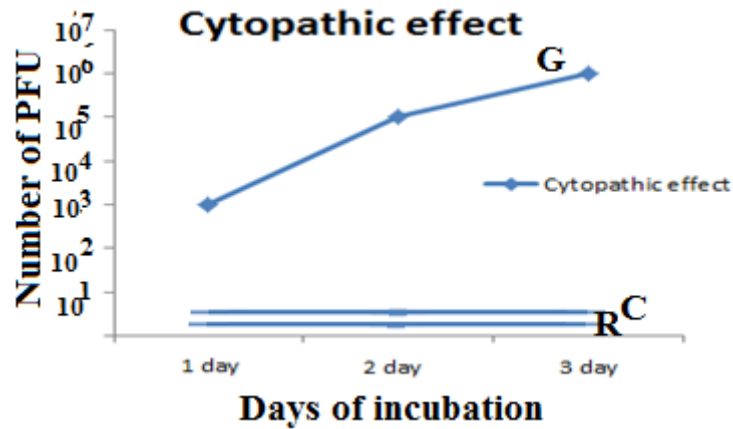| Collected samples and code number	Cytopathic effects (CPE)	Days of incubation
Conjunctivitis, (1 ,2, 3)	**No CPE detect**	**3 Day**
Respiratory (4, 5, 6)	**No CPE detect**	**3 Day**
Gastrointestinal (7, 8, 9, 10, 11, 12)	**CPE detect (10^6)**	**1-3 Day**

Fig (6): Histogram showing effect of adenovirus-induced cytopathic effects (PFU) on Vero cells inoculated with (**C**) conjunctivitis; (**R**) respiratory (**G**) Gastrointestinal samples.
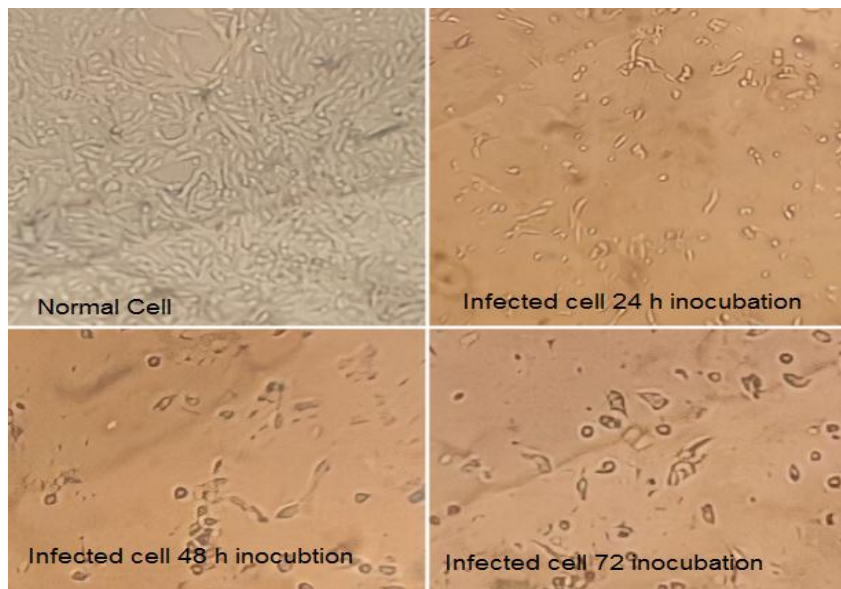

Fig (7): Vero cell lines inoculated with (**C**) conjunctivitis; (**R**) respiratory (**G**) Gastrointestinal samples showing effect of adenovirus-induced cytopathic effects (PFU)

HAdV isolates detection

The agarose gel (2%) electrophoresis was confirmed HAdV detection amplification by multiplex PCR. The agarose gel showed expected fragments size 350 bp of AdV Gastrointestinal samples (2, 3, 4) compared with HAdV positive control sample (1) as showing in (**Figure 8**).
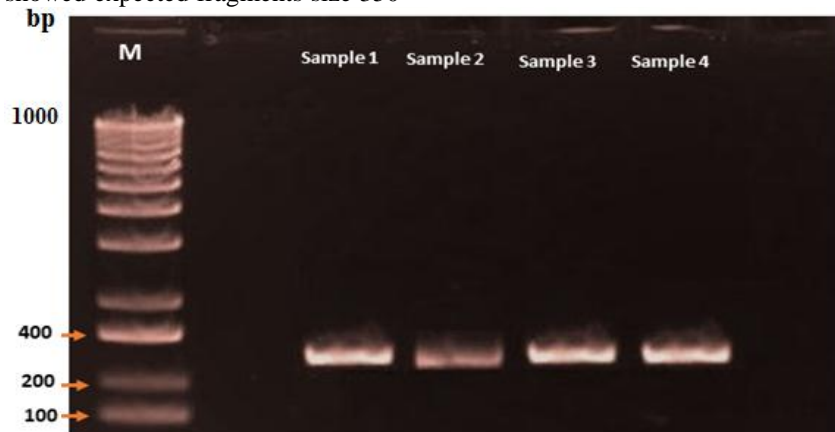
Fig (8): Electrogram of 2% agarose gel showing expected fragments size 350 bp of HAdV positive control sample (1) and HAdV Gastrointestinal samples (2, 3, 4) isolates obtained using multiplex PCR.

Genotype of HAdV isolates:

The three HAdV Gastrointestinal isolates belong to genotype F-40 as predicted from computerized analysis of the sequences was supported by phylogenetic comparison with 19 HAdV isolates published in GenBank. Evolutionary distances are calculated using the Kimura two-parameter method. All positions with gaps or missing data were removed.The four HAdV isolates have homogeneity of HAdV subtypes four different of HAdV subtypes and many more closely related Genotype F were identified by phylogenetic analysis (**Figure 9 and 10)** . HAdV sp. strain (HAdVF40/IAL-AD99/2015/BRA hexan protein. partial cds compared with sequence ID: MH201117.1 length : 362 Number of Matches :1

Fig (9): The genetic sequencing of three HAdV and control positive isolates showed positive for adenovirus compared with sequence ID: MH201117.1 length: 362 Number of Matches :1The genotypes that belong to genotype F, specifically serotype 40 of the four samples.

Classification of Viruses and Phylogenetic Relationships

Phylogeny of HAdV in comparison to the Tree of life (ToL). Bayesian phylogenies under the WAG nucleotide sequence substitution model with rate heterogeneity across sites and relaxed molecular clock with log-normal distribution of adoenviruses are drawn to a common scale of 0.2 nucleotide substitutions per position. Major lineages are indicated by vertical bars and names; arteri: Adenoiviridae, and sequence ID: MH201117.1 length: 362 Number of Matches :1. Support values at basal internal nodes are posterior probability support values. The combined set of distances was normalized relative to the largest distance that was set to one **(Figure 10).**
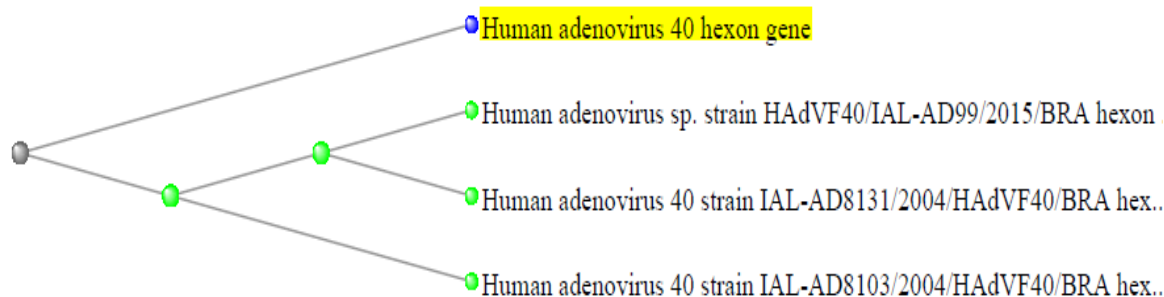
Fig (10): Phylogeny of HAdV in comparison to the Tree of life (ToL). Bayesian phylogenies under the WAG nucleotide sequence substitution model with rate heterogeneity across sites and relaxed molecular clock with log-normal distribution of adoenviruses are drawn to a common scale of 0.2 nucleotide substitutions per position.

Evaluation of diagnostic methods for HAdV:

That **(Table 6),** showing diagnostic methods including Clinical symptoms , Serological ,cell

culture and molecular detection .-It can be noticed that the results detected by (ILFST test) card was (20/90)cases giving positive result with probability of high false negative results, which confirmed by using cell culture ,which is more sensitive test than that of rapid cards that detecting 20/90 cases with positive result so giving false negative results -while multiplex PCR which is highly sensitive and morespecific technique than rapid cards result giving zero false negative result.

Table (6). Evaluation of diagnostic methods for (HAdV):

Diagnostic Methods	HAdenovirus characteristics						
	Antign	Antibodies	Genome	Coste	Time	Proficenality	Efficacy
Clinical Symptoms						***	
ILFST	*			****	****	*	>10 particle
Cell culture				*	*	***	>100 particle
Multiplex PCR			***	*	**	*****	More one particle

*Key (*****/Excellent. ****/Very good. ***/Good. ** and */ Acceptable).

Data analysis:

Statistical analysis of the data was performed using the statistical package of the social science software SPSS 26.0. Descriptive statistics were presented as frequencies, means and percentages. Statistically, significant differences were determined by Pearson's chi-square test. 0.05 was considered statistically significant.

Discussion

HAdV is one of the important causes of gastroenteritis, causing important symptoms such as diarrhea and fever, especially in people who suffer from immunodeficiency, as well as children under the age of five years. There are different genotypes of adenovirus that cause different infections. Among these genotypes that cause gastroenteritis are genotype F as well as genotype A and G (18). In the current study, we have used three different methods approaches for the detection of adenovirus, ILFST test, cell culture and multiplex PCR. ILFST test was done for all samples. Then the cell culture was inoculated into vero cell lines and often that confirmed through multiplex PCR. In ILFST test 20 (22.22 %) out of 90 stool samples, while the conjunctival and respiratory samples was negative. After that, cell culture was performed by Vero cells 12 samples , was confirmed in 6 samples (50%) through the appearance of cytopathic effect on the cells caused by viral infection. The molecular test was done to detect the genotypes of the adenovirus. 3 samples were positive by Multiplex PCR technique. The genotype of the 3 samples was belong to the genotype F serotype-40 (F-40) that causes gastroenteritis for the human. While a study in Egypt showed that the most prevalent serotype is serotype 41, followed by serotype 40, then serotype 6 and serotype 1(19).

Another study in Saudi Arabia showed that the most common serotype was genotype f-40, f-41, and these results are somewhat close to our results (20). Another study in the United States of America showed that the most common serotype is serotype 3, followed by serotype 2 and serotype 2. 1 and serotype 5, respectively, and this study is far from our findings (21). Another study in Denmark showed that the most prevalent serotype was the D genotype, with a small percentage of serotype 41, and this study differs with our results in terms of the higher prevalence (22). Another study in Brazil showed that the F genotype is the highest prevalence with a rate of 57.8%, specifically serotypes 40 and 41 where they were the highest prevalence, and this study is somewhat close to our study except for the prevalence rate in our study was 22.2% which is lower than prevalence in their studies (23). Our study also showed that males are more susceptible to infection than females, where the infection rate of males among the positive samples was 65% compared to 35% of the infection rate of females. A study conducted in Iran showed that males are more susceptible to infection with HAdV than females (24) and another study from Egypt that showed that males are more susceptible to infection than females (25). These two studies also agree with the findings of our studies.

Our studies showed that the age group from two to five years is the highest percentage in the number of infections, and this corresponds to another study conducted in Egypt, showed that children under five years are more likely to be infected (26). Other studies in Pakistan and Gabon showed that children under five years are more likely to be infected with HAdV (27-28). The difference between these studies and our results are due to the difference in the geographical location of the study site or the nature of the society and the economic situation of the country in which the study took place or the techniques used to detect the adenovirus and the extent of the efficiency of the researchers' work and these need studies More comprehensive and broader on a global scale.

Conclusion

This study provides baseline data About infections caused by HAdV such as gastroenteritis, respiratory system and conjunctivitis in different regions in Egypt. In summary, our study highlights

that adenoviruses circulate to a large extent in the community. As an important etiology of acute gastroenteritis with high disease burden. maybe this study does not provide a true picture of adenoviral gastroenteritis in Egypt. However, organizing systematic and comprehensive large-scale epidemiological investigations to understand the actual disease situation in Egypt has attracted the attention of medical professionals and policy makers, which is timely. intervention. In future studies, inclusion of multiple surveillance sites and examination of adenovirus genotypes in both rural and urban areas in Egypt and across the country will help us understand the true picture of adenoviral gastroenteritis in Egypt.

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