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Genotyping and interaction-reality of *Acanthamoeba*, enteric adenovirus and rotavirus in drinking water, Egypt.

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ABSTRACT

Access to safe water is essential to life and public health of human. Nearly, 884 million people about one-sixth of the world's populations are consuming contaminated water. In this study, the occurrence and the molecular characterization of enteric viruses particularly; enteric adenovirus, rotavirus and free-living protozoan *Acanthamoeba* spp. were determined in 180 tap water samples collected from five Egyptian governorates during December 2016 to November 2017. Moreover, we aimed to verify whether these isolated *Acanthamoeba* spp. could play a role in transmission of enteric adenovirus and rotavirus. Multiplex semi-nested RT-PCR, PCR and sequencing were used to detect and genotype rotavirus, enteric adenovirus and *Acanthamoeba*. Based on the sequence analysis of the Diagnostic Fragment 3 (DF3) of small subunit rRNA gene, *Acanthamoeba* isolates were genotyped. The present study showed that 26.7% (48/180) of tap water samples were positive for *Acanthamoeba* spp. The highest occurrence rate of *Acanthamoeba* (36.1%) was detected in tap water samples collected from Faiyum governorate. On the other hand, viral genomes were detected in 44 samples. Rotavirus and enteric adenovirus were observed in 15.6% (28/180) and 8.9% (16/180) of samples, respectively. Qalyubia and Faiyum governorates showed high level of viral contamination up to 50% (18/36) and 47.2% (17/36), respectively. Winter was the peak season for viral and *Acanthamoeba* contamination in tap water. Mixed contamination between at least one virus and *Acanthamoeba* was observed. *Acanthamoeba lenticulata*, *Acanthamoeba* T4, rotavirus G1P [8], and human enteric adenovirus 41 were the most predominant types identified during this work. The prevalence of pathogenic *Acanthamoeba* genotypes, especially genotype T4 and enteric viruses (rotavirus and adenovirus) in the examined tap water samples presented health hazards to consumers.

INTRODUCTION

Human enteric adenovirus and rotavirus have been reported as the main agents of acute gastroenteritis which is one of the leading causes of morbidity and mortality in children in the developing world (Oude Munnink and Van der Hoek, 2016).

These viruses are widespread in several natural and artificial water reservoirs worldwide. Despite all progress obtained in relation to environmental sanitation in the last century, the number of viral acute gastroenteritis resulting from consumption of contaminated drinking water is alarming. Human enteric adenovirus and rotavirus are recognized as important water contaminants (Iannelli *et al.*, 2014). These viruses are

typically transmitted by fecal-oral route, including contact with recreational marine water, contaminated swimming pools freshwater or tap water, as well as via inhalation of aerosolized droplets (Wyn-Jones *et al.,* 2011).

To date, fifty-two serotypes of adenovirus have been identified, one third of known genotypes are associated with human diseases such as gastroenteritis, respiratory infections, eye infections, acute hemorrhagic cystitis, and meningoencephalitis, while two thirds of genotypes are asymptomatic**.** About 5 to 15% of acute diarrheal infections in all continents are due to enteric adenovirus (Green, 2017; Dey *et al.,* 2011). Rotavirus are the main etiologic agent of diarrheal disease in children worldwide and are recognized as important environmental contaminants typically found in high concentrations in different matrices (Green, 2017). Nearly 3.9% of reported deaths in children are due to rotavirus infections (Neuzil *et al.,* 2010).

Acanthamoeba spp. are the most common free-living protozoa, isolated from public water supplies, swimming pools, bottled water, distilled water bottles, ventilation ducts, the water–air interface, air-conditioning units, soil, beaches, vegetables, air, surgical instruments, and contact lenses, indicating the ubiquitous nature of these organisms (Cruz and Rivera, 2014; Todd *et al.,* 2015; Al-Herrawy *et al.,* 2015). *Acanthamoeba* species can cause various diseases such as keratitis and granulomatous amoebic encephalitis, which is often fatal in immune-compromised as well as immunocompetent patients (Król-Turmińska and Olender, 2017). The possible mode of transmission includes direct contact of contaminated water, inhalation of cysts or trophozoites of *Acanthamoeba* carried by the wind through the respiratory tract, improper contact lens-care practices, or direct skin contact by traumatic injection or entry through preexisting wounds or lesions**.** To date, twenty different subtypes (T1–T20) of *Acanthamoeba* has been genetically characterized based on the variation of nucleotide sequences of the 18S rRNA gene (Corsaro *et al.,* 2015). Human enteric adenovirus, rotavirus and *Acanthamoeba* are being able to resist the environmental conditions; In addition they able to survive in water even after conventional physical-chemical treatment processes (Schuster and Visvesvara, 2004;Chen *et al.,* 2018). *Acanthamoeba* have been demonstrated the ability to serve as reservoirs for some viruses such as *coxsackie* B_3, adenovirus and mimivirus (Verani *et al.,* 2016; Raoult and Boyer, 2010). On opposite of this line, previous reports have been reported that *Acanthamoeba* not transmit poliovirus, vesicular stomatitis virus or echovirus (Danes and Cerva, 1981; Mattana *et al.,* 2006). The objectives of this study were to investigate the occurrence and the molecular characterization of adenovirus, rotavirus and representative free-living protozoan *Acanthamoeba* spp. in tap water in five Egyptian governorates. Moreover, to verify whether these isolated *Acanthamoeba* spp. could play a role in transmission of adenovirus and rotavirus or not.

MATERIALS AND METHODS

Sample Collection

A total of 180 tap water samples were collected from public café, restaurants and homes found in five separate cities; Cairo, Giza, Helwan, Qalyubia and Faiyum during December 2016 to November 2017. At each site, 21 liters of tap water were collected using sterilized 25-L plastic water storage containers. Samples were transported to the laboratory and processed at the same day of collection. One liter was used for parasitological analysis and 20 liters for viral analysis.

Parasitological Analysis

One liter of each sample was filtered through a nitrocellulose membrane (0.45µm pore size and 47mm diameter) using a stainless steel filter holder connected with a suction pump. Filtration was stopped just before drying of the membrane (APHA, 2005). After filtration process, the membrane was inverted face to face on the surface of a non-nutrient agar (NNA) plate seeded with heat-killed *Escherichia coli.* The plate was wrapped with parafilm and incubated at 30^{o}C for the cultivation of free living amoebae (APHA, 2005). Incubated plates were daily examined using inverted microscope for 7 days for the presence of any amoebic growth. Isolated and purified freshwater amoebae particularly *Acanthamoeba* were identified on the bases of both trophozoite and cyst morphological characteristics (Page, 1988). *Acanthamoeba* cysts walls were disrupted by three consecutive applications of freezing and thawing in liquid nitrogen, followed by incubation in a water bath at 100°C for 2 minutes. *Acanthamoeba* DNA was then extracted using the Qiagen QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA).

PCR was done to amplify a restricted fragment of DNA through generic primers (JDP1 and JDP2) for identification of *Acanthamoeba* species. Each PCR reaction was carried out in a final volume of 50 µl (25µl master mix "Promega, USA", 3 µl template DNA, 2 µl forward and reverse primers and 20 µl diethylpyrocarbonate "DEPC-treated water"). The amplification program included an initial denaturation at 95°C for 5 min, followed by 35 cycles; each consisted of denaturation at 94°C for 30 sec., annealing at 55°C for 40 sec and extension at 72°C for 40 sec. The program included a final extension step at 72°C for 10 min to generate amplification fragments from 450 to 500 bp (Schroeder *et al.,* 2001).

The positive PCR products were subjected to purify process using GeneJET PCR Purification Kit (Thermo Scientific, USA) according to the manufacturer instructions. The purified DNAs served as templates for DNA sequencing using the ABI PRISM® automated DNA Sequence. Nucleotide sequences were analyzed and assembled using the Laser gene 6 Package® (DNASTAR) and BLAST analysis tools (http://www.ncbi.nlm.gov/BLAST). The sequences were prepared and aligned using Clustal W implemented in the Bio-Edit program (version 7.0.4.1). Phylogenetic trees were constructed by neighbor-joining analysis with the Tamura-Nei model implemented in the MEGA6© program (Kumar *et al.,* 2016).

Virological Analysis

Each tap water sample (20 liter volume) was adjusted to acidified pH 3.5, after addition of $AlCl_3$ at a final concentration of 0.5 mM. Each sample was filtered using negatively charged nitrocellulose membrane (0.45 µm pore size, and 142 mm diameter). After filtration, 75 ml of 0.05 M glycine buffer, pH 9.5 containing 3% beef extract (Lab-Limco powder, Oxoid, UK) were added to eluted the adsorbed viruses according to Katzenelson *et al.* (1976).

An organic flocculation method was used for re-concentration of the eluted viruses. Briefly, each concentrated sample was acidified again to pH 3.5 and centrifuged at 3,000 rpm for 15 min. The pellet was dissolved in 1 ml of Na_2HPO_4 (0.14 N, pH 9). Concentrated samples subjected to DNA extraction using QiaAamp DNA Stool Mini Kit (QIAGEN, USA) and RNA extraction using GeneJET Viral DNA and RNA extraction kit (Thermo Scientific-USA) according to the manufacturers' instructions. The obtained pellet was dissolved in 40 µl of eluent and kept at –70°C until use. For detection and genotyping of human enteric adenovirus; according to Puig et al. (1994), the primers of Hexon gene [Forward primer Hex

AA1885 (5`- GCCGCAGTGGTCTTACATGCACATC-3`) and Reverse primer Hex1913 (5`- CAGCACGCCGCGGATGTCAAAGT-3`)] were used to amplify 300 bp amplicon. The PCR amplification was performed in a 25µl reaction mixture containing 5µl of DNA, 12.5µl of GoTaq G2 green master mix, (Promega, USA), forward and reverses primers at a concentration of 10 pmol. The conditions of PCR were 95°C for 3 min as an initial denaturation step, followed by 40 cycles of 95°C for 20 sec, 55°C for 30 sec, and 72°C for 30 sec and completed by 72^{o}C for 10 min. Gel electrophoresis was done using 1.5% agarose to analyze the PCR products. According to Pring-Akerblom and Adrian (1994), second amplification was performed as the pervious reaction except using specific primer sequences (Table 1) to genotype the positive samples of adenovirus. The PCR conditions were performed in 40 cycles of 94°C for 60 sec, 45°C for 30 sec and 72°C for 40 sec, followed by the extension step continued for 10 min at 72°C. PCR products were visualized using 1.5% agarose gel.

Table 1: Sequence of primers for detection and genotyping of enteric adenovirus.

Virus	Position	Name	Sequence	Length
	302- 321	H1	5'-TTGACATCCGCGGCGTGCTG-3'	
Ad40	1240-1221	H40	5'-TATTCTGAGACCAGTTAGTT-3'	939
Ad4l	1460-1441	H41	5'-CTGCAGTCCAGGTTTGGCCA-3'	942

For detection and genotyping of human rotavirus, specific primers for VP6 segment were used to amplify 382bp and 147bp amplicons by using nested RT-PCR according to Gray and Iturriza-Gómara (2011). After that, Cocktail of primers were selected to genotype rotavirus based on the characterization of VP7 and VP4 genes into G-type and P-type using multiplex semi-nested RT-PCR, respectively (Gray and Iturriza-Gómara, 2011; Gouvea *et al.,* 1994a; Gouvea *et al.,* 1994b). P and G genotypes were determined according to amplicon size (Table 2).

Table 2: Sequence of primers for genotyping of VP7 and VP4 genes of rotavirus.

Primer name	Sequence 5´–3´	Sense	Target gene	Ref.	Primer set	Amplicon Length
VP7-F	ATGTATGGTATTGAATATACCAC	+	9 (VP7)			
VP7-R	AACTTGCCACCATTTTTTCC	-	9 (VP7)		VP7-F/VP7-R	881
aBT1	CAAGTACTCAAATCAATGATGG	+	9 (VP7)		aBT1/VP7-R	618
aCT2	CAATGATATTAACACATTTTCTGTG	+	9 (VP7)		aCT2/VP7-R	521
G3	ACGAACTCAACACGAGAGG	+	9 (VP7)	(Gray and	G3/VP7-R	682
aDT4	CGTTTCTGGTGAGGAGTTG	+	9 (VP7)	Iturriza-	aDT4/VP7-R	452
G8	TTRTCGCACCATTTGTGAAAT	+	9 (VP7)	Gomara, 2011)	G8V/P7-R	756
G9	CTTGATGTGACTAYAAATAC	+	9 (VP7)		G9/ VP7-R	179
G10	ATGTCAGACTACARATACTGG	+	9 (VP7)		G10/VP7-R	266
FT5	CATGTACTCGTTGTTACGTC	_	9 (VP7)		VP7-F/ FT5	729
DT6	CTAGTTCCTGTGTAGAATC	_	9 (VP7)	(Gouvea *et al.,*	VP7-F/ DT6	449
BT11	GTCATCAGCAATCTGAGTTGC	_	9 (VP7)	1994a)	VP7-F/ BT11	286
VP4-F	TATGCTCCAGTNAATTGG	+	4 (VP4)			
VP4-R	ATTGCATTTCTTTCCATAATG	-	4 (VP4)		VP4-F/ VP4-R	663
2T-1	CTATTGTTAGAGGTTAGAGTC	-	4 (VP4)		VP4-F/2T-1	483
3T-1	TGTTGATTAGTTGGATTCAA	-	4 (VP4)		VP4-F/3T-1	267
1T-1D	TCTACTGGRTTRACNTGC	-	4 (VP4)	(Gray and	VP4-F/1T-1D	345
4T-1	TGAGACATG CAATTGGAC	-	4 (VP4)	Iturriza-	VP4-F/4T-1	391
5T-1	ATCATAGTTAGTAGTCGG	-	4 (VP4)	Gomara, 2011)	VP4-F/5T-1	583
P(11)	GTAAACATCCAGAATGTG	-	4 (VP4)		VP4-F/ P(11)	312
pNCDV	CGAACGCGGGGGTGGTAGTTG	+	4 (VP4)		pNCDV /VP4-R	526
pUK	GCCAGGTGTCGCATCAGAG	+	4 (VP4)	Gouvea *et al.,*	pUK /VP4-R	459
pOSU	CTTTATCGGTGGAGAATACGTCAC	+	4 (VP4)	1994b)	pOSU /VP4-R	406

Testing the presence of enteric viruses in *Acanthamoeba* spp.

The presence of human enteric adenovirus or rotavirus was tested in the amoebic culture of all isolated strains using PCR and RT-PCR as follow; the isolated *Acanthamoeba* strains were subjected to subculture on non-nutrient agar seeded with *E. coli* and were examined regularly to observe trophic growth. The trophozoite of each *Acanthamoeba* strain was subjected to disrupt via three cycles of freezing and thawing. Finally, cell debris was removed by centrifugation (Mattana *et al.*, 2006). The obtained solutions after trophozoites' disruption were used for extracting RNA and DNA using GeneJET Viral DNA & RNA extraction kit (Thermo Scientific-USA) according to manufacturer instructions. The obtained RNA and DNA were stored at -70^{o}C until PCR processing for adenovirus and rotavirus as previously mentioned.

RESULTS

Totally, the present study showed that human adenovirus and rotavirus group A (RVA) genomes were found in 8.9% (16/180) and 15.6% (28/180) of water samples. The highest occurrence for rotavirus was in 30.6% (11/36) of samples collected from Qalyubia governorate, followed by 25% (9/36) in Faiyum governorate. For enteric adenovirus, the highest rate was in Faiyum (22.2%) and Qalyubia (19.4%). Cairo is the only site which was negative for both viruses while Giza governorate was positive for rotavirus (11%) and negative for enteric adenovirus (Table 3). *Acanthamoeba* spp. was identified in 48 samples with percentage up to 26.7% using morphological and molecular assays. Again, Faiyum governorate was the highest site for occurrence of *Acanthamoeba* in tap water 36.1% (13/36), followed by Helwan 27.8% (10/36). Cairo was the lowest site for occurrence of *Acanthamoeba* 19.4% (7/36). Furthermore, co-infections were observed in at least two governorates such as Faiyum and Qalyubia that represented the highest sites for co-infections (Table 3).

Table 3: Occurrence of rotavirus, enteric adenovirus and *Acanthamoeba* in Egyptian's governorates.

Pathogen	Faiyum	Qalyubia	Helwan	Giza	Cairo	Total
Rotavirus	(09/36)	(11/36)	(04/36)	(04/36)	(00/36)	(28/180)
	25%	30.6%	11%	11%	00%	15.6%
Adenovirus	(08/36)	(07/36)	(01/36)	(00/36)	(00/36)	(16/180)
	22.2%	19.4%	03%	00%	00%	08%
Acanthamoeba	(13/36)	(09/36)	(10/36)	(09/36)	(07/36)	(48/180)
spp.	36%	25%	27%	25%	19%	26.7%
Acanthamoeb-	(02/36)	(03/36)	(00/36)	(01/36)	(00/36)	(06/180)
Rota	06%	08%	00%	03%	00%	3.3%
Acanthamoeb-	(03/36)	(02/36)	(00/36)	(00/36)	(00/36)	(05/180)
Aden	08%	06%	00%	00%	00%	2.8%

Concerning seasonal variations, winter followed by autumn was the peak for viral and *Acanthamoeba* spp. contamination in all governorates. For example, in Faiyum governorate (the highest site for the presence of 3 pathogens in this study), winter was the highest season for occurrence of *Acanthamoeba* spp., rotavirus and enteric adenovirus with percentages up to 55.5% (5/9), 66.6% (6/9) and 66.6% (6/9), respectively. Qalyubia governorate as well, winter was the highest season for occurrence of *Acanthamoeba* spp. (33.3%), rotavirus (100%) and enteric adenovirus (66.6%). In general, 40%, 46% and 26% of all positive samples for *Acanthamoeba* spp., rotavirus and enteric adenovirus observed in winter season, respectively (Table 4). Although all pathogens have been identified throughout the year, wet seasons showed the highest occurrence for all pathogens during this work.

Table 4: Seasonal distribution of three pathogens in five governorates in Egypt.

Governorate	Pathogen	Winter	Spring	Summer	Autumn
Cairo	**Rotavirus**	0/9	0/9	0/9	0/9
	Adenovirus	0/9	0/9	0/9	0/9
	***Acanthamoeba* spp.**	2/9	1/9	3/9	1/9
Giza	**Rotavirus**	3/9	0/9	0/9	1/9
	Adenovirus	0/9	0/9	0/9	0/9
	***Acanthamoeba* spp.**	4/9	1/9	3/9	1/9
Helwan	**Rotavirus**	3/9	0/9	0/9	1/9
	Adenovirus	0/9	0/9	0/9	1/9
	***Acanthamoeba* spp.**	4/9	2/9	2/9	2/9
Qalyubia	**Rotavirus**	9/9	0/9	0/9	2/9
	Adenovirus	6/9	0/9	0/9	1/9
	***Acanthamoeba* spp.**	3/9	2/9	1/9	3/9
Faiyum	**Rotavirus**	6/9	0/9	0/9	3/9
	Adenovirus	6/9	0/9	0/9	2/9
	***Acanthamoeba* spp.**	5/9	3/9	3/9	2/9
Total	**Rotavirus**	(21/45) 46%	(0/45) 00%	(0/45) 00%	(7/45) 15%
	Adenovirus	(12/45) 26%	(0/45) 00%	(0/45) 00%	(4/45) 09%
	***Acanthamoeba* spp.**	(18/45) 40%	(9/45) 20%	(12/45) 26%	(9/45) 20%

On the other hand, genotyping for all pathogens was done by using PCR or sequencer. For *Acanthamoeba* spp., 47.9 % (23/48) of positive isolates were genotyped using sequencing. Totally, the most predominant types found in all governorates except Cairo were *Acanthamoeba lenticulata* (18.8%) and T4 (10.4%). The distribution of genotypes was differed from one governorate to another. For example, In Cairo, 57.2% was identified for both T9 and T15, while in Faiyum governorate, 23.1%, 15.4%, and 7.7% were identified for *A. lenticulata*, T5, and T4, respectively.

In Qalyubia governorate, *A. lenticulata* was the predominant species with occurrence rate more than 33.3% followed by T4 (11.1%). In Helwan, T4 and T3 were the most common genotypes while *A. lenticulata* was the only common species identified based on sequencing data in Giza (Figures 1&2).

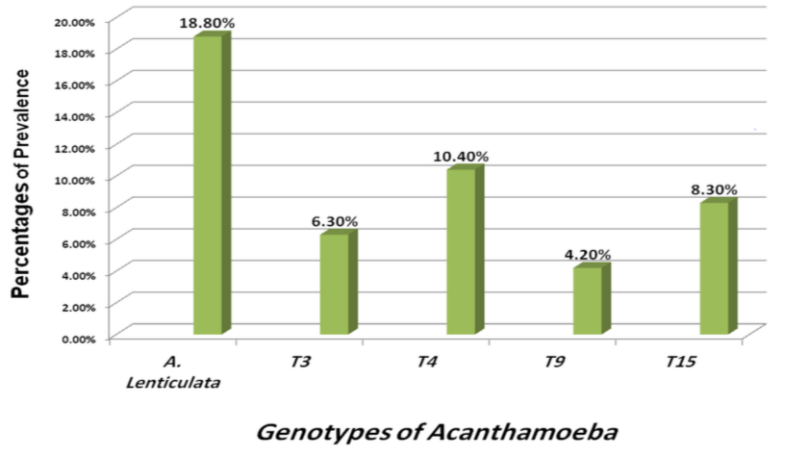

Fig. 1: Total Prevalence of *Acanthamoeba* genotypes in tap water in Egypt.

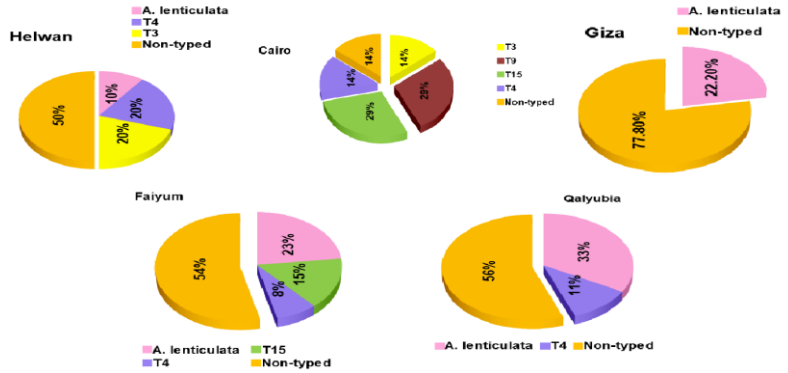

Fig. 2: The prevalence of *Acanthamoeba* genotypes in tap water in each governorate.

The sequences of our *Acanthamoeba* isolates (Egy1 - Egy5) showed high similarity percentages up to 99-100% with genotype T4 which have Accession numbers such as MF576064.1, MH938698.1, JN399021.1. While *Acanthamoeba* isolates (Egy6 - Egy8) were identical to genotype T3 which has Accession numbers such as KY613497.1 and KT215188.1. *Acanthamoeba* isolates (Egy9 - Egy12) and (Egy13 - Egy21) showed 100% of similarity to *Acanthamoeba* genotype T15 and *Acanthamoeba lenticulata*. Finally, two isolates of *Acanthamoeba* [Egy isolate (22) and Egy isolate (23)] revealed 99% similarity with *Acanthamoeba* genotype T9: Accession number KU936116.1 as shown in Figure (3). The accession numbers of our sequences of the isolated *Acanthamoeba* spp. were deposited in the GenBank under accession numbers; MK217498, MK217499, MK217500, MK217501, MK217502, MK217503, MK217504, MK217505, MK217506, MK217507, MK217508, MK217509, MK217510, MK217511, MK217512, MK217513, MK217514, MK217515, MK217516, MK217517, MK217518, MK217519, MK217520.

Fig. 3: Phylogenetic tree of *Acanthamoeba* isolates from Egypt. The maximum-likelihood (ML) constructed phylogenetic tree from *Acanthamoeba* isolates and reference SSU rRNA gene

sequences from GenBank showing the phylogenetic position of the 4 different genotypes (T3,T4, T9 and T15) and *Acanthamoeba lenticulata*.

For adenovirus, the most predominant genotype found in all governorates was enteric adenovirus type 41. Furthermore, enteric adenovirus type 40 was identified also in 14 % of collected samples from Qalyubia governorate. For rotavirus, G1P[8] was the most predominant genotype found in more than 50 % of all collected samples in each site. About 78 %, 75 %, 55 % and 50 % of collected water samples were positive for G1P[8] in Faiyum, Giza, Qalyubia and Helwan governorates, respectively. Furthermore, G2P[8], G2P[4], G4P[8] and G9P[8] genotypes were also identified (Figures 4&5).

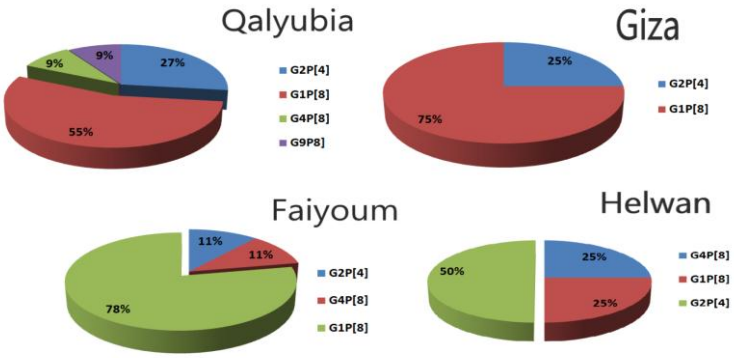

Fig. 4: The prevalence of rotavirus genotypes in tap water of Egypt.

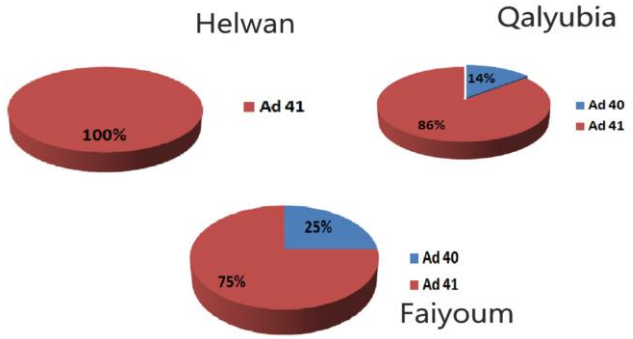

Fig. 5: The prevalence of enteric adenovirus genotypes in tap water of Egypt.

Finally, all isolated *Acanthamoeba* strains were tested by using PCR for presence of viral genome (rotavirus& adenovirus) inside the amoebic trophozoites. The obtained results showed that no viral genes were detected in any amoebic trophozoites for rotavirus or adenovirus in all isolated *Acanthamoeba* strains.

DISCUSSION

Acanthamoeba spp. are the most common free-living amoebae have been isolated from a wide range of environments particularly water. Free-living amoebae have been reported to feed by phagocytosis on bacteria, fungi, and algae (Król-Turmińska and Olender, 2017; Chen *et al.*, 2018). According to the previous reports

(Scheid and Schwarzenberger, 2012; Yousuf *et al.,* 2017), *Acanthamoeba* might serve as an environmental reservoir for viruses living in the same environment, such as *mimivirus, coxsackievirus* and adenovirus. In light of the fact that viruses can become internalized within *Acanthamoeba*, this is the first study to investigate the transmission of adenovirus and rotavirus via waterborne' *Acanthamoeba* in Egypt. River Nile is the main source for drinking water supplied to population of the investigated five governorates in Egypt. Raw water is carried from river into water treatment plants for settling, filtration and chlorination. After that, drinking water is distributed to local populations of these governorates. Totally, 26.6%, 15.6% and 8.9% out of 180 tap water samples were positive for *Acanthamoeba* spp., rotavirus and enteric adenovirus, respectively. When our findings were grouped per each governorate, it was remarkable that tap water of two governorates (Cairo and Giza) out of five showed no adenoviral contamination and only one governorate (Cairo) was clean from any viral contamination.

However, tap water of all governorates were positive for *Acanthamoeba* spp. with percentages up to 36%, 27%, 25%, 25% and 19% in Faiyum, Helwan, Qalyubia, Giza and Cairo, respectively (Table 3). Faiyum followed by Qalyubia governorate markedly showed the highest contamination rates for three pathogens either separately or co-infection. For example, out of 36 water samples collected from Faiyum, 36%, 25% and 22% were positive for *Acanthamoeba,* rotavirus and enteric adenovirus, respectively. Several studies conducted previously in Egypt, reported that 80%, 58.6%, 56.3%, 31.4%, 67.7% and 29.2% of water samples were positive for *Acanthamoeba* spp. in Beni-Suef governorate, Nile Delta governorates, Giza governorate, Cairo governorate and Faiyum governorate, respectively (Abd El Wahab *et al.,* 2018; Al-Herrawy *et al.,* 2017; Morsy *et al.,* 2016; Tawfeek *et al.,* 2016; Sakran *et al.,* 2017; Gad and Al-Herrawy, 2016). Globally**,** *Acanthamoeba* spp. have been documented in tap water including Korea (5.8%) Nicaragua (19%), Turkey (4.4% and 26.8%) and Philippines (9.1%) (Jeong and Yu, 2005; Leiva *et al.,* 2008; Coşkun *et al.,* 2013;Onichandran *et al.,* 2014). In general there are big differences in detection rates of *Acanthamoeba* in different sites and countries may due to the difference in geographic areas, the quality of raw water sources or additional treatment technologies facilities in each country.

Our findings for rotavirus were relatively similar to the previous reports conducted in Egypt (8.3%), South Africa (14.9) and Brazil (15.7%) (Rizk and Allayeh, 2018; Chigor and Okoh, 2012; Miagostovich *et al.,* 2014) and disagree with the previously reports conducted globally in drinking water samples such as; China (22.4%), Ghana (48%), and Italy (37.5%) (Glass *et al.,* 1996; Sumi *et al.,* 2013; Grassi *et al.,* 2010). As well our results for enteric adenovirus were very low in contrast with the previous studies conducted in sewage (10.4%) and stool samples (6.7%) collected from Egypt according to previous studies (Kamel *et al.,* 2010; Allayeh *et al.,* 2018) and globally as West Africa (12.9%) (Verheyen *et al.,* 2009). These comprehensive data for identification the prevalence of different pathogens in waters from several governorates in Egypt indicated the low prevalence of viral contamination in contrast with *Acanthamoeba* contamination.

The incidence of rotavirus infections rates were high in the summer season in the USA (Gutiérrez-Aguirre *et al.,* 2008) and Northern Asian regions (He *et al.,* 2009). In moderate climate regions, colder and drier months of the year were peaked (Ansari *et al.,* 1991). For enteric adenovirus, there is no significant seasonal variation globally (Lin *et al.,* 2000). The present study indicated the wet seasons (winter and autumn) were the peak for rotavirus and adenovirus. This result was similar with the

previous report conducted in Egypt about the incidence of both rotavirus and adenovirus (Allayeh *et al.,* 2018).

The seasonal variation of *Acanthamoeba* was noted, with a peak during summer months or warmer months either in clinical or water samples (Page and Mathers, 2013; Gad and Al-Herrawy, 2016). Our results for *Acanthamoeba* spp. were not similar to the previous reports because we found their incidence throughout the year in all governorates. Fifty-one genotypes were identified for adenovirus and classified into six species (A to F), enteric adenovirus serotypes 40 and 41 are the unique members of the species F (Green, 2017). Twenty-seven and thirty-seven of G- and P-genotypes for human rotavirus were identified, respectively (Trojnar *et al.,* 2013). The most common human RVA genotypes circulating in the worldwide were G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] (Santos and Hoshino, 2005). In the present study, adenovirus type 41 (Ad41) and rotavirus G1P[8] and G2P[4] were the most common predominant genotype identified in the examined tap water and uncommon genotypes were also identified such as G4P[8] and G9P[8]. The results were completely in agreement with the previous studies reported that Ad41, G1P[8] and G2P[4] were the most common genotypes for adenovirus and rotavirus in Egypt, respectively (Kamel *et al.,* 2010; El-Senousy *et al.,* 2013; Rizk and Allayeh, 2018; Allayeh *et al.,* 2018).

For genotyping of *Acanthamoeba* spp., currently twenty genotypes of the genus *Acanthamoeba* were identified on the basis of variation in nucleotide sequences of the 18S rRNA gene (Fuerst *et al.,* 2015). The findings concluded that *Acanthamoeba lenticulata* and genotype T4 were identified as the most common genotypes found in tap water of five governorates. Furthermore, other genotypes such as T5, T15, T3 and T9 were identified during this work (Figures 1-3). Our findings are completely in agreement with the previous reports demonstrated that various *Acanthamoeba* genotypes isolated from different countries such as T3 and T5 were isolated from swimming pools in Egypt (Morsy *et al.,* 2016), T1-4 and T7 were isolated from freshwater in Egypt (Lorenzo-Morales *et al.,* 2006), T3 and T4 were isolated from water in France (Ovrutsky *et al.,* 2013), T3-5 and T11 were isolated from tap water in Iran (Niyyati *et al.,* 2015) and T4, T5 and T16 were isolated from soil and tap water in China (Xuan *et al.,* 2017).

Many reports have been existed about viruses' internalized protozoa (Verani *et al.,* 2016; La Scola and Raoult, 2001; Alotaibi, 2011), while viruses/protozoa (e.g *Acanthamoeba*) relationship is less well understood. Previous reports have been indicated that *Acanthamoeba* not internalize poliovirus, vesicular stomatitis virus or echoviruses (Danes and Cerva, 1981; Mattana *et al.,* 2006). Recently, Mimivirus and adenovirus were identified from *Acanthamoeba* (Scheid and Schwarzenberger, 2012; Yousuf *et al.,* 2017). Because *Acanthamoeba* and adenovirus or *Acanthamoeba* and rotavirus, could be detected in the same water sample, we hypothesized that there might be a role for *Acanthamoeba* in transmission of adenovirus or rotavirus. In light of the fact that viruses can become internalized inside *Acanthamoeba*, we tested the forty-eight isolates of *Acanthamoeba* spp. for the presence of human adenovirus and human rotavirus genomes using molecular methods exclusively. Our findings indicated that a total of forty-eight of *Acanthamoeba* strains have no internalization for tested viral genomes. These findings are in contrast to the pervious findings (Mattana *et al.,* 2006; Alotaibi, 2011). However, the mixed infections between *Acanthamoeba*/rotavirus and *Acanthamoeba*/adenovirus were observed in five and six water samples, respectively. Based on our findings, the isolated *Acanthamoeba* could not be considered as a potential carrier for freely particles of adenovirus or rotavirus.

CONCLUSION

The relatively high prevalence of *Acanthamoeba* and enteric viruses in tap water presents a public health hazards which highlights the importance of surveying the water sources in Egypt. Rotavirus G1P[8], adenovirus type 41 *Acanthamoeba* genotype T4 were the most predominate genotypes in tap water in Egypt. Generally, this work has underlined the need for additional studies in rural areas in Egypt to investigate the relationship between viruses and *Acanthamoeba* spp.

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CONFLICT OF INTEREST

The authors declared that there are no conflicts of interest.

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ARABIC SUMMARY

التوصيف الجزيئي وحقيقة علاقة الاكنثاميبا *(Acanthamoeba)* و الفيروسات المعوية الروتا والادينو (rotavirus & adenovirus) في مياه الشرب بمصر.

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١- معمل طفيليات البيئة - قسم بحوث تلوث المياه - المركز القومى للبحوث ، الجيزة، مصر.

٢- معمل فيروسات البيئة - قسم بحوث تلوث المياه - المركز القومى للبحوث ، الجيزة، مصر.

إن الحصول على المياه الصالحة للشرب أمر ضروري للحياة والصحة العامة للبشر. ما يقرب من ٨٨٤ مليون شخص حوالى سدس سكان العالم يستهلكون المياه الملوثة. الدراسة الحالية تهدف إلى التوصيف الجزيئى لعزلات الاكنثاميبا *Acanthamoeba* و الفيروسات المعوية الروتا والادينو rotavirus & adenovirus و التحقق مما إذا كانت هناك علاقة بين عزلات الاكنثاميبا *Acanthamoeba* و الفيروسات المعوية الروتا والادينو rotavirus & adenovirus فى مياه الشرب. فى الدراسة الحالية تم تجميع ١٨٠ عينة مياه شرب من خمس محافظات فى مصر خلال الفترة من ديسمبر ٢٠١٦ حتى نوفمبر ٢٠١٧. تم استخدام تقنية تفاعل البلمرة المتسلسل المتعدد العكسى (Multiplex RT-PCR) والتفاعل االمتسلسل Sequencing لتوصيف الفيروسات المعوية و الاكنثاميبا ، على التوالى. أظهرت نتائج الدراسة الحالية وجود عزلات الاكنثاميبا بنسبة ٢٦.٧ ٪ (١٨٠/٤٨) من عينات مياه الصنبور. ظهر أعلى معدل انتشار للأكنثاميبا (٣٦.١٪) في عينات ماء الصنبور التي تم جمعها من محافظة الفيوم. من ناحية أخرى ، وجدت جينات الفيروسات المعوية في ٤٤ عينة مياه شرب. وقد لوحظ فيروس الروتا rotavirus و فيروس الادينو adenovirus في ١٥.٦ ٪ (١٨٠/٢٨) و ٨.٩ ٪ (١٨٠/١٦) من عينات مياه الشرب المختبرة، على التوالي. أظهرت النتائج ان محافظتي القليوبية والفيوم بهما اعلى نسبة تواجد للفيروسات المعوية بنسبة تصل إلى ٥٠٪ (٣٦/١٨) و ٤٧.٢٪ (٣٦/١٧)، على التوالي. و كان فصل الشتاء موسم الذروة لانتشار الفيروسات المعوية و الاكنثاميبا في مياه الصنبور. لوحظ وجود تلوث مختلط بين فيروس واحد على الأقل و الاكنثاميبا. أما عن الانواع الاكثر انتشار فى هذة الدراسة هى الاكنثاميبا لينتيكولاتا *Acanthamoeba lenticulata* و الاكنثاميبا *Acanthamoeba* T4 و rotavirus G1P[8] ، والادينو adenovirus 41. فى النهاية، انتشار انواع مختلفة من الاكنثاميبا وخاصة المسببة للأمراض مثل النمط الجيني genotype T4 و الفيروسات المعوية الروتا و الادينو في عينات مياه الشرب تعتبر تهديدا للصحة العامة للانسان.