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Original Research

Existence and Removal of Rotaviruses Group A and *Cryptosporidium* Species in a Wastewater Treatment Plant

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Abstract

Rotavirus group A (RV-A) and *Cryptosporidium* spp. are recognized as important pathogens causing gastroenteritis, even with very low doses in all age groups and representing a public health risk. Seventy-two wastewater samples were collected and concentrated for detecting *Cryptosporidium* spp. and RV-A. PCR was performed for identification of genus *Cryptosporidium* using generic primers and species specific primers for *Cryptosporidium parvum* / *Cryptosporidium hominis*. Multiplex semi-nested RT-PCR was performed to identify the genotypes P and G of RV-A. Our findings showed that the rotavirus genome was detected in 29 (40.3%) out of 72 analyzed wastewater samples. Rotavirus rates in raw wastewater samples were higher (47.2%) than those detected in treated wastewater (33.3%). *Cryptosporidium* was found in 33.3% of 72 tested wastewater samples. All *Cryptosporidium*-positive-samples were related to *Cryptosporidium parvum*. The prevalence of *Cryptosporidium parvum* in influents and effluents reached 41.7% and 25%, respectively. The removal rates of rotavirus and *Cryptosporidium parvum* in the examined WWTP were 29.4% and 40%, respectively. The largest percentage of positive rotavirus in raw wastewater samples was detected in winter (77.8%), followed by 66.7% in autumn. Three P types and two G types of RV-A strains were identified in wastewater samples. The most prevalent types of RV-A strains in raw and treated sewage samples were G1 and P[8] genotypes. In conclusion, wastewater treatment processes, including a trickling filter, were not sufficient to eliminate several RV-A strains and *Cryptosporidium parvum*, leading to the potential release of these pathogens from domestic sewage into water sources.

Keywords: rotavirus genotypes, *Cryptosporidium* spp., removal, wastewater, multiplex RT-PCR

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Introduction

A protozoan *Cryptosporidium* is a parasite that infects various animal species and humans [1]. Its zoonotic potential as well as its ubiquitous presence in numerous animal species and the environment is confirmed. Globally, *Cryptosporidium* is a major contributor of diarrheal diseases in young farm animals and causes about 20% of diarrheal episodes in children of developing countries [2, 3]. The largest documented waterborne parasitic disease outbreak in history is attributed to this organism and to date it remains refractory to all conventional treatments. *Cryptosporidium* is also extremely resistant to disinfection [2]. Comparatively, PCR techniques are more sensitive and specific than conventional diagnostic microscopy for the detection of *Cryptosporidium* in clinical and environmental samples [4]. Many species of *Cryptosporidium* are of little public health significance, however the vast majority of human cryptosporidiosis is caused by *Cryptosporidium parvum* and *Cryptosporidium hominis* [2]. Worldwide, severe gastroenteritis caused by human rotaviruses group A are responsible for about 600,000 deaths annually in infants and children [5]. In developed countries, rotaviruses are considered a common cause of morbidity with a considerable economic burden [6]. Children 5 years old are most susceptible to rotavirus infection, and reinfection may occur during life [6]. The genome of rotaviruses is composed of double helix molecules of RNA with 11 segments, encoding six viral proteins (VPs) that form the virus particle and six nonstructural proteins (NSPs) [6]. Rotaviruses group A are classified according to genes encoding the outer capsid proteins (glycoprotein) which define G type and protease-cleaved proteins which define P genotypes. About 37 P and 27 G rotavirus genotypes have been detected in humans and animals [7]. Only a limited number of GxP[y] genotypes confederations are popular in humans, such as G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8][7], and unusual combinations such as G8P[4], G12P[8], G3P[9], G10P[8], G10P[14] and G1P[4] have also been reported lately [8-10].

The transmission of *Cryptosporidium* and rotavirus occurs by the consumption of contaminated water and food or from person to person through fecal-oral route. These pathogens are stable in the environment for a long period of time. After replication in the gastrointestinal tract, viruses are shed at very high concentrations of up to 10^{10} viruses/g in feces [3, 6]. The main purpose of advanced wastewater treatment is to eliminate/reduce pathogen levels in order to prevent their environmental transmission. For the removal of suspended solids, pathogenic microorganisms and organic materials, several physical, biological, and chemical wastewater treatment processes are used. The nature of pathogenic microorganisms determine their removal/reduction efficiency by the wastewater treatment process [11]. Rotavirus and *Cryptosporidium*, as other enteric pathogens, are resistant against the processes used in wastewater treatment plants, and thus lead to their spread into the environment [12], particularly in surface waters. However, RV-A have been implicated in waterborne gastroenteritis outbreaks only sporadically [13]. Individuals with either symptomatic disease or asymptomatic infection excreted rotaviruses in sewage [14]. Monitoring of pathogens in urban sewage is therefore useful to evaluate the potential threatening viruses/protozoa widespread in the population, independent of disease severity and subject age. Monitoring of RV-A in sewage of WWTPs may provide an additional means to assess genotypes that also circulate in a given population [15]. However, detection and genotyping of RV-A in sewage may be affected by the simultaneous presence of several common or uncommon strains, and the segmented nature of the rotavirus genome may preclude the definite identification of the full genome constellation of detected RV-A. So, the objectives of the present study were to assess the occurrence and removal efficiency of *Cryptosporidium* species and rotavirus in the tested WWTP and identify the species of *Cryptosporidium* and RV-A genotypes.

Materials and Methods

Samples and Sampling Site

This study was carried out on a wastewater treatment plant (WWTP) located in Sharkeya governorate, Egypt, and operated by trickling filter technology. The designed capacity of the wastewater treatment plant is 12500 m^3/day. For preliminary treatment in the examined wastewater treatment plant a bar screen is installed to remove large objects such as sticks, cans and debris that may cause flow obstructions during the collection of domestic wastewater. FOG removal chambers contain grease, sand, oil traps and interceptors that are used to prevent these wastes from entering the plant and causing problems downstream. A primary sedimentation tank generally removes 30 to 40% of total BOD and 50 to 70% of suspended solids from raw sewage through anaerobic treatment. Trickling filters are used, in which an attached-growth biological process occurs through an inert medium to attract microorganisms that form a film on the medium surface. A rotatory or stationary distribution mechanism distributes wastewater from the top of the filter percolating it through the interstices of the film-covered medium. As the wastewater moves through the filter, the organic matter is adsorbed onto the film and degraded by a mixed population of aerobic microorganisms. The oxygen required for organic degradation is supplied by air circulating through the filter induced by natural draft or ventilation. A secondary sedimentation basin is a chamber in which microorganisms and other solids

are settled, part of activated sludge is pumped back into an aeration basin and excess activated sludge is pumped to thickeners and then to a drying basin. The treated effluent is injected with a final concentration of 0.5-1 ppm chlorine to destroy pathogenic microbes. A total of 72 water samples were collected from inlets (n = 36) and outlets (n = 36) of a wastewater treatment plant during a one-year period from July 2016 to June 2017. Three- and two-liter samples were collected in sterile bottles for viral and parasitological analysis, respectively. The removal rates (R) of the WWTP were calculated as the following equation;

$$\mathbf{R} = \frac{\textbf{Total removed in effluents}}{\textbf{Positve samples in influents}} \times \mathbf{100} \quad [16].$$

Samples Concentration and Processing

For virological examination, wastewater samples were separately concentrated by filtration through negatively charged nitrocellulose membranes (0.45 µm pore size) after the addition of $AlCl_3$ to a final concentration of 0.5 mM and acidification to pH 3.5, and after passing through Whatmann No. 1 filter paper. The adsorbed viruses to the membrane were eluted with 75 ml of 0.05M glycine buffer having pH 9.5 (using HCl 5N) and containing 3% beef extract (Lab-Limco powder, OXOID, UK) [17]. All samples were reconcentrated using an organic flocculation method according to Katzenelson et al. [18]. The eluate was acidified to pH 3.5 using HCl (5N), centrifuged at 3000rpm for 15min, the supernatant was discarded and the pellet was dissolved in 1 ml of Na_2HPO_4 (0.14 N, pH 9). Samples were neutralized and kept at -70ºC until used [19].

For protozoological examination, wastewater samples (2L volume each) were concentrated by centrifugation at 4000xg at 4ºC for 30min (Sigma 3-18KS Centrifuge, Germany). The obtained pellets were separately resuspended in PBS (pH 7.4) and subsequently centrifuged for 15 min at 4300xg at 4ºC. The final pellet was purified using zinc sulfate solution (specific gravity, 1.3) according to Moodley et al. [20]. After purification, the obtained sediment was suspended in 280µl buffer AL (Qiagen, Hilden, Germany) for DNA extraction.

Nucleic Acid Extraction

Viral nucleic acids were extracted from 200 µl of the concentrated sample using GeneJET Viral DNA and RNA Purification kit (THERMO SCIENTIFIC-USA) according to the manufacturer's instructions. The obtained RNA was dissolved in 40µl of eluent and kept at -70ºC until used.

DNA of *Cryptosporidium* oocysts was extracted from in the purified samples using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Three freeze-thaw cycles, each cycle consisting of 2 min in liquid nitrogen followed by 2 min in boiling water, were performed before the application of the manufacturer's protocol. The obtained DNA was stored at −20ºC until used.

Detecting *Cryptosporidium* spp. by PCR

Cryptosporidium primers were described by Spano et al. (1997) [21], forward primer Cry-9 (5`-GGACTGAAATACAGGCATTATCTTG-3`) and reverse primer Cry-15 (5`-GTAGATAATGGAAGAGATTGTG-3`) to amplify *Cryptosporidium* spp. cowp gene. Species identification was performed on PCR-positive samples for *Cryptosporidium* by using species-specific primers for *Cryptosporidium parvum* (SB012/SB012) and *Cryptosporidium hominis* [22, 23]. Amplification of DNA was performed using GoTaq G2 Green Master Mix (Promega, USA) according to the manufacturer manual. PCR reaction mixture per sample consisted of 12.5 µl master mix, 3 µl template DNA, 1 µl of forward and reverse primers (conc. 10 pmol), and 8.5 µl nuclease-free water. PCR products were visualized using 2% agarose gel stained with ethidium bromide.

Detecting Rotavirus Group A Using VP6

According to Gray and Iturriza-Gomara [24], Nested RT-PCR was used for the detection of VP6 segment of rotavirus using the forward primer VP6-F primer 5'-GACGGVGCRACTACATGGT-3' and reverse primer VP6-R 5'-GTCCAATTCATNCCTGGTG-3' to amplify 382bp in first RT-PCR. Then the forward primer, VP6-NF 5'-GCTAGAAATTTTGATACA-3', and the reverse primer, VP6-NR 5'-TCTGCAGTTTGTGAATC-3', were used to amplify 155bp in the second PCR. PCR products were analyzed on 2% agarose gel.

Rotavirus Genotyping

The multiplex semi-nested RT-PCR method was performed for genotyping of RV-A in water samples based on the characterization of VP7 and VP4 genes into G-type and P-type, respectively [24-26]. The cocktail of the primers in this method allowed us to determine G1-G6, G8-G11, P[1], and P[4]-P[11] types (Table 1). All PCR products were examined using 2% gel electrophoresis. P and G genotypes were determined according to amplicon size.

Results and Discussion

Wastewater treatment steps consist of physical treatment (through sedimentation and filtration), biological treatment (trickling filters, stabilization ponds and activated sludge) and inactivation by chlorine, ozone and chlorine dioxide. The effluents of wastewater treatment plants may be contaminated by various groups of pathogenic microorganisms such as viruses, protozoan parasites, bacteria, and parasitic worms.

Table 1. Primer sequences 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)	[24]		
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)		G3/VP7-R	682
aDT4	CGTTTCTGGTGAGGAGTTG	+	9 (VP7)		aDT4/VP7-R	452
G8	TTRTCGCACCATTTGTGAAAT	+	9 (VP7)		G8V/P7-R	756
G9	CTTGATGTGACTAYAAATAC	+	9 (VP7)		G9/ VP7-R	179
G10	ATGTCAGACTACARATACTGG	+	9 (VP7)		G10/VP7-R	266
FT5	CATGTACTCGTTGTTACGTC	_	9 (VP7)	[25]	VP7-F/ FT5	729
DT6	CTAGTTCCTGTGTAGAATC	_	9 (VP7)		VP7-F/ DT6	449
BT11	GTCATCAGCAATCTGAGTTGC	_	9 (VP7)		VP7-F/ BT11	286
VP4-F	TATGCTCCAGTNAATTGG	+	4 (VP4)	[24]		
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)		VP4-F/1T-1D	345
4T-1	TGAGACATG CAATTGGAC	-	4 (VP4)		VP4-F/4T-1	391
5T-1	ATCATAGTTAGTAGTCGG	-	4 (VP4)		VP4-F/5T-1	583
P(11)	GTAAACATCCAGAATGTG	-	4 (VP4)		VP4-F/ P(11)	312
pNCDV	CGAACGCGGGGGTGGTAGTTG	+	4 (VP4)	[26]	pNCDV /VP4-R	526
pUK	GCCAGGTGTCGCATCAGAG	+	4 (VP4)		pUK /VP4-R	459
pOSU	CTTTATCGGTGGAGAATACGTCAC	+	4 (VP4)		pOSU /VP4-R	406

Therefore, it is necessary to prevent environmental emissions of these pathogens through the application of the inactivation process on effluents before discharging it in surface water or use for irrigation [11]. Overall, the prevalence rates of rotavirus in influent (n = 36) and effluent (n = 36) samples of the examined WWTP were 47.2% and 33.3%, respectively. These data were comparable with the results of previous studies showing the presence of RV-A not only in raw, but also in treated wastewater [27, 28]. In the present study the rotavirus genome was detected in 29 (40.3 %) out of 72 wastewater samples (raw and treated). An active laboratory monitoring and evaluation of the RV-A circulating genotypes will be required for surveilling the RV-A genetic diversity as well as their substantial characteristics, such as the seasonal fluctuations and the generation of new combinations or novel strains due to interspecies barrier broken [9]. Higher proportions were found in different countries like Tunis (72.4%) [27], Venezuela (67%) [29], Argentina (91.4 and 100%) [28, 30], Brazil (85%) [31], Italy (60.4%) [32], USA (58.3%) [33], and Uruguay (52.6%) [34], but a lower prevalence of RV-A was observed in Egypt (8.3%) [35], and China (32.3%) [36]. The differences in environmental factors, virus levels in populations, methodology, primers used. might lead to different results. Also, the possibility of the presence of RNA inhibitors in environmental samples might also interfere with molecular detection [37]. There have only been limited data on the removal by trickling filter wastewater treatment process. In the current study, the removal efficiency of the virus was 29.4%, which agreed with previous reports in other parts of the world, suggesting that even properly working wastewater treatment systems removed only 20-80% of enteric viruses [38,39] and confirming the high resistance of the virus to the sewage treatment process. Another environmental study showed that the removal of group A rotaviruses was 20% in two different wastewater treatment plants (trickling and activated sludge) [33].

Species-specific primers for *Cryptosporidium hominis* and *Cryptosporidium parvum* were applied on all positive samples for genus *Cryptosporidium*. The obtained results proved that all the samples were

Table 2. Prevalence of rotavirus and *Cryptosporidium parvum* in influent and effluent of the examined WWTP.

Sample types	Collected samples	Rotavirus			*Cryptosporidium parvum*		
		No.	Prevalence (%)	Removal (%)	No.	Prevalence (%)	Removal (%)
Influent	36	17	47.2%	29.4	15	41.7	40
Effluent	36	12	33.3%		9	25	
Total	72	29	40.3		24	33.3	

related to *Cryptosporidium parvum*. The prevalence of *Cryptosporidium parvum* in influents and effluents was 41.7% and 25%, respectively. Other studies showed that the prevalence of *Cryptosporidium* oocysts ranged from 6.4 to 100% in raw wastewater from Brazil, China, Germany, Spain and USA [40-44]. The prevalence rates of *Cryptosporidium* oocysts varied in various studies even in the same country [37]. These differences in the prevalence rates might be due to the differences in the detection methods, sample volumes, number of the collected samples and the loads of the infection with *Cryptosporidium* in a given community.

Total removal rates in the examined WWTP were 29.4% and 40% for rotavirus and *Cryptosporidium parvum*, respectively (Table 2). Globally, rare studies were performed about the evolution of *Cryptosporidium* removal through trickling filters of wastewater treatment plants. Stadterman et al. [45] found that the activated sludge was better than trickling filters for the removal of seeded *Cryptosporidium* oocysts. Also, the reduction of *Cryptosporidium* from effluents was inefficient by using chlorine disinfection and by trickling filter [11]. Moreover, UV irradiation and ultrafiltration as tertiary treatment processes were able to reduce *Cryptosporidium* oocysts in secondary effluents [46, 47]. The importance of efficient wastewater treatment is to protect water bodies that receive the treated wastewater effluents from pathogen transmission [11].

The highest percentage of positive rotavirus in raw wastewater samples was detected in winter (77.8%), with a maximum existence in December (100%), then in autumn (66.7%). These data were in agreement with the results of a previous study in Tunis showing that the highest prevalence of RV-A was in winter [27]. The highest percentage of positive samples for *C. parvum* (66.7%) was observed in summer in influent samples, followed by 55.6, 33.3 and 11.1% in spring, autumn and winter, respectively. Other reports from

Table 3. Seasonal variations and genotyping of rotavirus group A in inlet and outlet of the examined wastewater treatment plant.

Season	Month	Occurrence of Rotavirus in inlet	+ve G-Type in inlet	+ve P-Type in inlet	Occurrence of Rotavirus in outlet	+ve G-Type in outlet	+ve P-Type in outlet
Summer	June	33.3% (1/3)	1	1	0% (0/3)	ND	ND
	July	0% (0/3)	ND	ND	0% (0/3)	ND	ND
	Aug.	33.3% (1/3)	1	1	0% (0/3)	ND	ND
	Total	22.2% (2/9)			0% (0/9)		
Autumn	Sep.	66.7% (2/3)	1	1	66.7% (2/3)	1	1
	Oct.	66.7% (2/3)	ND	ND	66.7% (2/3)	ND	ND
	Nov.	66.7% (2/3)	2	1	66.7% (2/3)	2	1
	Total	66.7% (6/9)			66.7%(6/9)		
Winter	Dec.	100% (3/3)	ND	ND	66.7% (2/3)	ND	ND
	Jan.	66.7% (2/3)	1	1	33.3% (1/3)	ND	ND
	Feb.	66.7% (2/3)	2	2	66.7% (2/3)	2	2
	Total	77.8% (7/9)			55.6% (5/9)		
Spring	March	33.3% (1/3)	1	ND	33.3% (1/3)	1	ND
	April	33.3% (1/3)	1	1	0% (0/3)	ND	ND
	May	0% (0/3)	ND	ND	0% (0/3)	ND	ND
	Total	22.2% (2/9)			11.1% (1/9)		
Total	47.2% (17/36)		58.8% (10/17)	47.1% (8/17)	33.3% (12/36)	50% (6/12)	33.3% (4/12)

ND: *Not detectable*

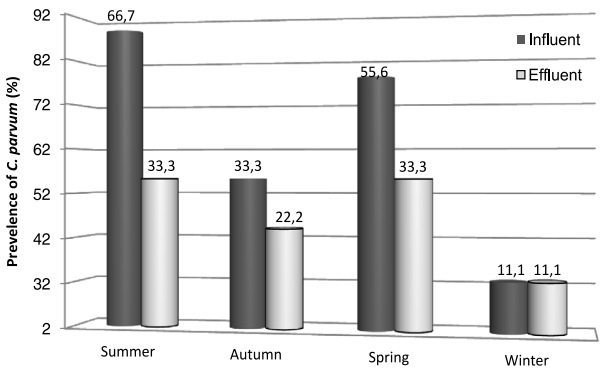

Fig. 1. Seasonal variations of *C. parvum* in influent and effluent of the examined WWTP.

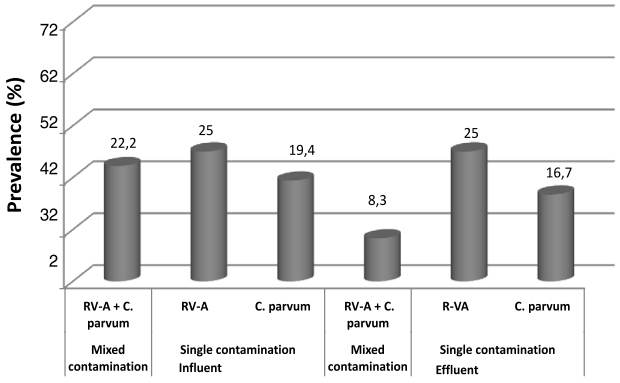

Fig. 2. Single and mixed contamination with rotavirus and *C. parvum*.

different countries showed that the seasonal distribution of *Cryptosporidium* oocysts were predominant during autumn and winter in Germany [48], spring and summer in Spain [49], and summer, autumn, and winter in Ireland [50].

The single contamination with rotavirus in raw sewage samples was 25%, while that of *C. parvum* was 19.4%. The rates of single contamination with rotavirus and *C. parvum* in effluent samples were 25 and 16.7%, respectively. The mixed contamination with both rotavirus and *C. parvum* in influent and effluent samples reached 22.2 and 8.3%, respectively (Table 3, Figs 1-2). Unfortunately, no data about mixed contamination with rotavirus and *C. parvum* in wastewater were available.

Rotaviruses G and P Types

Ten of the total 17 detected rotavirus strains in raw sewage could be G typed, while 41% of the samples were untypeable. G typing of RV-A strains showed 2 different types: G1 in 8 samples (47% prevalence), and G3 in 2 samples (12%). At the same time, 6 out of 12 rotavirus strains detected in treated wastewater could be G typed, while 50% of the positive-samples for rotavirus were untypeable. The most frequent G type in treated sewage samples positive for rotavirus was G1 (41.7%) (5/12), followed by G3 (8.3%) (1/12). Also, No mixed contamination with G types was noticed in any sewage samples (Table 3, Fig. 3). In the raw sewage samples, 8 of 17 (47%) rotavirus-containing samples could be P typed, while 53% of the samples were untypeable. The most frequent rotavirus P type was P[8] (35.2%) (6/17), followed by P[6] (5.9%) (1/17) and P[4] (5.9%) (1/17). In the treated wastewater samples, 4 of 12 (33.3%) rotavirus-positive samples could be P typed, and thus 66.7% of the samples were untypeable. The frequencies of P types were P[8] (25%) (3/12) and P[4] 8.3% (1/12). Rotavirus types P[1], P[5], P[7], P[9], P[10] and P[11] were not detected throughout the current study (Table 3, Fig. 3). In the current study, the prevalence of G1 and P[8] genotypes in sewage samples was in agreement with the predominance of G1 and P[8] genotypes in sewage

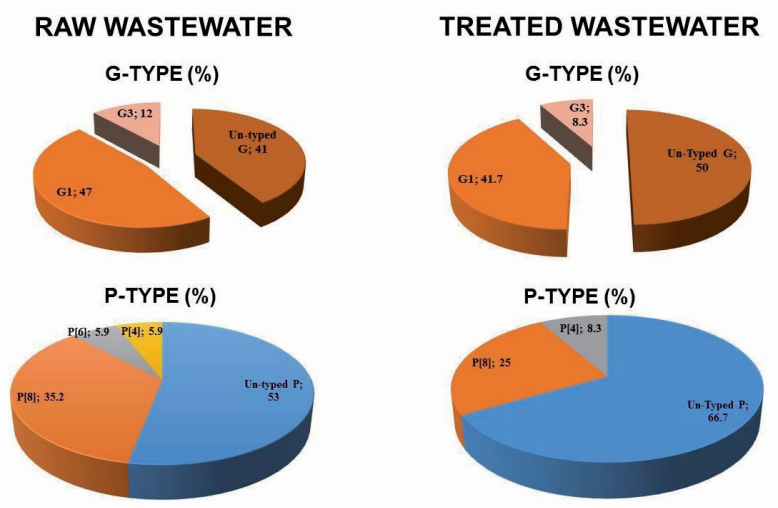

Fig. 3. Distribution of rotavirus genotypes in raw and treated wastewater samples.

samples in other study [27]. Another environmental study reported the same genotypes in children suffering from diarrhea and in sewage at the same time [51]. In different countries, researchers found that G1 and P[8] genotypes were dominant in wastewater samples [27, 35, 36]. In the present study, other genotypes (P[4] and G3) were also detected. Genotypes G2 and P[4] were detected in Tunis, but with relatively high frequency in sewage samples [27]. The study conducted in Argentina showed approximate frequencies of the different genotypes (G2, G3, G4, G8, G9) in sewage and stool samples [30]. In another study conducted in Italy, other G types G2, G9, G4, G6, G3, and G26; and P types P[4], P[6], P[9], P[14], and P[19] were found [32].

Conclusions

Several pathogenic strains of RV-A and *Cryptosporidium parvum* were not efficiently eliminated by trickling filter wastewater treatment processes, leading to the potential release of these pathogens from domestic sewage into water sources. The most prevalent types of RV-A strains in raw and treated sewage samples were G1 and P[8] genotypes. The virulent *Cryptosporidium parvum* was detected in all *Cryptosporidium*-positive samples. Concerning seasonal variations, the highest distribution rates for rotaviruses were in winter, while for *Cryptosporidium* species were in summer. This study also demonstrated the usefulness of environmental surveillance as an additional tool to determine the circulation of *Cryptosporidium* species and RV-A in a certain community. Large-scale studies are required to investigate the circulation of enteric pathogens in the Egyptian community, and the removal of these pathogens by different treatment technologies.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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