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Molecular detection of three gastroenteritis viruses in an urban sewage treatment plant and river water in Egypt

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ABSTRACT
In this study, the prevalence of astrovirus (AstV), norovirus (NoV), and group C rotaviruses (RVC) were described in urban sewage, sewage sludge, river water, and sediment samples from Egypt. AstV, NoV, and RVC were detected in 58.3%, 33.3%, and 25% of the raw sewage samples; in 33.3%, 25%, and 16.6% of the treated sewage samples; and in 66.6%, 16.6%, and 8.3% of the sludge samples, respectively. On the other hand, AstV, NoV, and RVC were detected in 25%, 16.6%, and 8.3% of the river water samples and in 16.6%, 8.3%, and 0% of the river sediment samples, respectively. The study revealed that AstV genogroup B and NoV genogroup I are the most frequent genotypes in the Egyptian environment. Furthermore, the peak prevalence of AstV and RVC in river water and raw sewage samples was found in winter months however there was no clear seasonality for NoV spread. This study support the importance of considering viral markers to ensure the quality of water and the utilization of these markers as additional tests for the characterization of water contamination.

INTRODUCTION

Enteric viruses are identified as the most common cause of acute gastroenteritis in human. Enteric viruses possess specific properties that enable them to be easily found in wastewater and aquatic environment, including high-level fecal shedding and resistance to harsh environmental factors such as temperature, sunlight, and pH. Their persistence in various aquatic environments also depends on the viral composition, such as the viral genome (DNA or RNA), protein capsid structure, and the virus ability to attach to suspended solids (Fong and Lipp, 2005).

Astroviruses (AstVs), belonging to the Astroviridae family, are recognized as one of the most major causes of infantile diarrheal disease, worldwide (Guix *et al*., 2005). Large outbreaks of gastroenteritis due to AstV infections are being documented with increasing frequency (Oishi *et al*. 1994; Hwang *et al*., 2015). Based on genetic and antigenic properties, HAstVs are divided into 2 genogroups A and B; genogroups A includes 1 to 5 and 8 serotypes whereas genogroup B includes 6 and 7 serotypes (Belliot *et al*., 1997). Previous studies reported that AstV-1 is the most common type worldwide (Noel *et al*. 1995, Guix *et al*. 2002, Silva *et al*. 2006).

Noroviruses (NoVs), belonging to the Caliciviridae family, are considered as a major pathogen of sporadic cases and outbreaks of acute diarrhea in human (Patel *et*

al. 2009). NoVs are classified genetically into 7 genogroups (NoV GI–GVII) based on the complete sequence of capsid gen nucleotides (De Graaf *et al*. 2015; Vinje, 2015); of these, NoV GI, NoV GII and NoV GIV are associated with human gastroenteritis (Vinjé, 2015). NoV GII is the most prevalent genogroup and is usually associated with acute gastroenteritis outbreaks (Matthews *et al*. 2012). However, NoV GI has been found with a higher positivity than NoV GII in some environmental studies (Kitajima *et al*. 2010; La Rosa *et al*. 2010; Moresco *et al*. 2012).

Rotaviruses are also considered important causes of diarrhoeal diseases among infants and young children, worldwide. Rotaviruses, belonging to the family Reoviridae, are divided into seven serogroups (RVA-RVG), based on genetic and antigenic properties (Saif and Jiang, 1994), of which group A, B, and C are identified to infect both humans and animals (Bridger *et al*., 1987). Several sporadic diarrhoeal illnesses and diarrheal outbreaks in human due to group C rotaviruses infection have been reported in several studies, worldwide (Khamrin *et al*. 2008; Moon *et al*. 2011; Araújo *et al*. 2011; Tiku *et al*. 2017; Joshi *et al*., 2017).

AstVs, NoVs, and RVC have been detected in different types of water, such as drinking water (Kukkula *et al*. 1999; El-Senousy *et al*. 2014), sewage (Meleg *et al*. 2008; Kitajima *et al*. 2012, El-Senousy *et al*., 2014), seawater (Yokoi et al., 2001;Victoria *et al*. 2014), rivers (Kitajima *et al*. 2010; El-Senousy *et al*. 2015; Prevost *et al.* 2015) and recreational waters (Taylor *et al*.,2001; Vieira *et al*. 2012). Because these viruses can be excreted at a high concentration (10^9 - 10^{15} particles/g) in the feces of infected patients (Zhang *et al*. 2006; Ludwig *et al*. 2008), investigation of municipal wastewater samples could be an effective tool to understand the actual frequency and distribution of these viruses (Gofti-Laroche *et al*. 2003; Scarcella *et al*. 2009). In this research, we investigated the prevalence of AstVs, NoVs, and group C rotavirus in water of Rosetta River Nile and wastewater of a large WWTP which discharges its effluents via El-Rahawy drain into this River Nile.

MATERIALS AND METHODS

Sample collection

During the period from October 2017 to September 2018, 36 samples (12 raw sewage, 12 treated sewage, and 12 sewage sludge) were collected from Abu-Rawash WWTP. In addition, 24 samples (12 river water and 12 river sediment) were collected from Rosetta River Nile after mixing with El-Rahawy wastewater during the period from April 2017 to March 2018. At each collection, one sample from each type was systematically collected monthly. All the samples were stored at 4 ^{o}C upon arrival in the laboratory. Abu-Rawash WWTP provides only a primary treatment and its effluent moves along several drains until reaching Rosetta River Nile via El-Rahawy drain (Figure 1), thus this WWTP is considered as a major cause of water quality degradation at the Rosetta branch.

Virus concentration methods

Viral concentration in river water and wastewater samples

Viral concentrations in the wastewater and river water samples were performed by using a protocol described by katayama et al. (2002), with slight modification. The sample was mixed with 2.5 M $MgCl_2$ (5 ml of $MgCl_2$/500 ml sample) then filtered through a negatively charged membrane (0.45 µm pore size and 142 mm diameter). Filter membrane was rinsed with 200 mL of 0.5 mM H_2SO_4 solution. The membrane was removed and transferred to sterile glassware then eluted in 10 mL of 1 mM NaOH (pH=10.5). After stirring for 10 min, the eluate was neutralized by adding 50

µL 1 mM Tris-EDTA buffer solution (pH 8.0) and 50 µL of 50 mM H_2SO_4 solution. After that, the neutralized eluate was ultra-filtrated by using a Centriprep Concentrator system then the filtrate was centrifuged at 1,500 xg for 10 min at 4°C. After removing the filtrate, the pellet was rinsed twice with 10 ml of double distilled water by the same procedure, and a final volume of 2 ml was obtained. The concentrated samples were stored at −80°C until the next analysis.

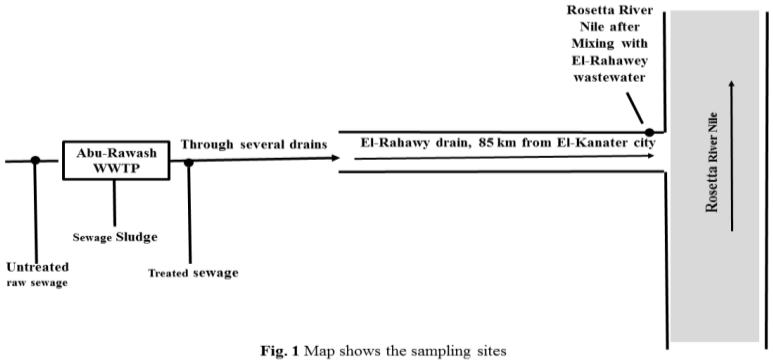

Fig. 1 Map shows the sampling sites

Viral Concentration in Sediment and sludge Samples

Viral concentrations in the sediment and sludge samples were carried out according to a protocol previously described by EPA, (1992), with some modifications as documented by Schlindwein et al. (2010). In summary, 10 ml of 5 M $AlCl_3$ solution was added to 20 grams of the sample and the pH of the mixture was adjusted to 3.5 using 5M HCl. After stirring for 30 min at 500 rpm, the mixture was subjected to centrifugation for 15 min at 2,500 x*g* and 4°C then the pellet was dissolved in 35 ml of 10% beef extract buffer (PH=7). After additional stirring for 30 min at 500 rpm, centrifugation step for 30 min at10,000 *xg* and 4°C was done. The resulting supernatant was collected and its pH was adjusted to 7 when required. For the concentration of virus, PEG 6000 precipitation method was performed as documented by Lewis and Metcalf (1988). The supernatant was transferred to clean tube containing 15% PEG 6000 (pH 7.2) and after stirring for about 2 h, final centrifugation was done at 1000 χg for 20 min at 4°C. The resulting pellet was suspended in 10 mL of 0.1 M PBS (pH 7) then treated with penicillin (100 units/ml) and streptomycin (100µg/ml) for decontamination.

Molecular detection of NoV and AstV, and RVC

RNAs extraction and cDNAs synthesis

Nucleic acids were extracted from 140 µL of the eluate to obtain a final volume of 60 µL, using the QIAamp Viral RNA (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

The synthesis of complementary DNA (cDNA) was conducted using 5µl of extracted RNA mixed with 1.5µl of 10x reaction buffer, 0.5µl of RNase inhibitor, 4µl of 2.5mM dNTPs (Promega), 1µl of primer (A2 primer for AstV genogroup A, A2 bis primer for AstV genogroup B, G1-SKR primer for NoV GI, G2-SKR primer for NoV GII, or random primer for RVC), 0.5µl of Maxima Reverse Transcriptase Thermo scientific (200 U/µL), and 2.5 µl of double distilled water (DDW). The RT of AstV was carried out at 42°C for 60 min, and the RT of NoV was performed at 25 °C for 10 min, 42°C for 60 min, followed by an enzyme inactivation step at 99 °C for 5 min, then the tubes were held at 4°C for 5 min or until PCR amplification, whereas the RT of RVC was conducted at 37℃ for 60 min, followed by heating step at 95℃ for 5 min to an enzyme inactivation, and immediate cooling at 4℃.

Semi nested-RT PCR amplification of AstV, NoV, and RVC

The AstV cDNA was amplified using a semi-nested RT-PCR method. For first-PCR, 15µl of the synthesized cDNA was mixed with 35 µl of PCR reaction containing 4 µl of the 10x reaction buffer, , 4 µl of 2.5mM dNTPs, 0.5 µl of A1 and A2 primers for AstV-A or A1 bis and A2 bis for AstV-B, 0.5µl of Taq DNA polymerase, and 26 µl of DDW. For nested-PCR, 2 µl of the first PCR products were mixed with 5 µl of 10x reaction buffer, 4 µl of 2.5mM dNTPs, 1 µl of A1 and A2 internal primers for AstV-A or A1 bis and A2 internal primers for AstV-B, 0.5 µl of the Taq DNA polymerase, and 36.5 µl of DDW. The PCR program of the first and semi-nested RT-PCR was consisted of 3 min at 95°C followed by 40 cycles of 30 sec at 94°C, 30 s at 55°C, 30 sec at 72°C, and a final extension step at 72°C for 7 min (Guix *et al.* 2002; El-Senousy *et al.* 2007).

On the other hand, amplification of NoV cDNA by semi nested-RT-PCR was performed using the same reagents and concentrations used with AstV. For NoV GI, COG1F primer was used in first PCR while G1-SKF primer was used in second PCR. For NoV GII, COG2F primer was used in first PCR whereas G2-SKR primer was used in second PCR. The PCR conditions used in first PCR were as follows: 94 ^{o}C for 3 min, followed by 40 cycles of amplification (94 ^{o}C for 1 min, 50 ^{o}C for 1 min, and 72 ^{o}C for 2 min), then final incubation at 72 ^{o}C for 15 min. The same cycling conditions were applied in semi-nested RT-PCR with 35 cycles (Kojima *et al.* 2002; Kageyama *et al.* 2003).

Also, RVC was amplified using the same reagent and concentrations described above with using FP-1 and BMJ-13 primers in first PCR. The semi-nested RT-PCR (518 bp) was conducted using the first round product as the template with using forward BMJ-107 and reverse BMJ-13 primers. The thermal profile for the first round and semi-nested RT-PCR was 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 48 °C for 2 min and 72 °C for 3 min and final extension at 72 °C for 7 min (Jiang *et al.* 1995; Sanchez-Fauquier *et al.* 2003). All primer sequences used in this study and the final PCR product size are presented in Table 1.

Table 1: The sequence of primers used for detection of astrovirus, noroviryus, and group C rotavirus by qualitative PCR

Virus	Sequence (5`-3`)	Amplicon size (bp)	References
Astrovirus genogroup A			
A1	CCTGCCCCGAGAACAACCAAGC	192-237 depending on the strain	Guix et al. (2002); El-Senousy *et al*. (2007)
A2	GTAAGATTCCCAGATTGGTGC		
A2 internal	CCATACGTTTGTTTGTGAGTATGG-		
Astrovirus genogroup B			
A1bis	CCTGCCCCCCGTATAATTAAAC	167	
A2bis	ATAGGACTCCCATATAGGTGC		
A2 internal	CCATACGTTTGTTTGTGAGTATGG		
Norovirus GI			
COG1F	CGYTGGATGCGNTTYCATGA	330	Kojima et al. (2002); Kageyama *et al*. (2003)
G1SKR	CCAACCCARCCATTRTACA		
G1SKF	CTGCCCGAATTYGTAAATGA		
Norovirus GII			
COG2F	CARGARBCNATGTTYAGRTGGATGAG	344	
G2SKR	CCRCCNGCATRHCCRTTRTACAT		
G2SKF	CNTGGGAGGGCGATCGCAA		
Rotavirus group C			
VP7 FP-1	GGC ATT TAA AAA AGA AGA AGC TG	518	Jiang *et al*. (1995); Sanchez-Fauquier *et al*. (2003)
BMJ-13	AGC CAC ATG ATC TTG TTT		
BMJ-107	TGT TTG GAG ATG TGA TGA		

RESULTS AND DISCUSSION

Human enteric viruses are shed in the feces of infected individuals and are mainly transmitted by ingestion of fecally contaminated food and/or water, the surveillance of virus circulating in the environment is significant in order to well understand the virus epidemiology and to identify the sources of viral contamination. The objective of this study was to determine the prevalence of some enteric viruses in the Egyptian environment with studying the impact of Abu-Rawash WWTP effluents on Rosetta River for one year. In this study, the prevalence of AstV, NoV, and group C rotavirus was evaluated in wastewater, sewage sludge, river water, and river sediment samples collected from Abu Rawash WWTP and the Rosetta River Nile. In order to increase specificity of detection, prevent false-positive results, and enhance the amplification efficiency, semi-nested RT-PCR amplification was used for the detection of these viruses in all samples. This strategy enabled us to detect the viral contaminants at small numbers in the tested samples.

The detection rates of AstV, NoV, and RVC in untreated and treated sewage samples collected from Abu-Rawash WWTP are displayed in Figure 2. The number of AstV, NoV, and RVC positive samples were decreased upon treatment in this WWTP, but the reduction rates were not statistically significant. Viruses could be detected in 10/12 (83.3%) and 6/12 (50%) of raw and treated sewage, respectively, suggesting that the current WWTP reduced 40% of viral positivity. This data indicates that the biological treatment applied in this WTTP has limited effect on the occurrence of these viruses. AstV, NoV, and RVC were found in 7/12 (58.3%), 4/12(33.35), and 3/12(25%) of raw sewage samples, respectively, whereas they were found in 4/12(33.3%), 3/12(25%), and 2/12(16.6%) of treated sewage samples, respectively. In activated sludge samples, AstV, NoV, and RVC were found in 8/12(66.6%), 2/12(16.6%), 1/12(8.3%), respectively.

Similar studies from Egypt stated a higher prevalence of astrovirus in raw sewage (68.6 -73.7%) and treated sewage (26.3 - 34.3%) (El-Senousy et al., 2014 and 2015). Also, a study conducted in Hungary on astrovirus showed a relatively low prevalence in raw sewage (43%) (Meleg *et al*. 2006). The incidence of NoV and RVC detected in the current study is higher than the incidence detected by Kamel *et al*. (2010) and El-Senousy et al. (2014 and 2015) in raw and treated sewage from three sewage treatment plants in Egypt. However, La Rosa *et al*. (2010) detected about 100 % of positivity for NoV in the raw sewage of five WWTPs in Italy and Meleg *et al*. (2008) detected RVC in 91% and 57% of raw and treated sewage of four WWTPs in Hungary, respectively.

River waters in many countries, including Egypt, receive the treated wastewater from wastewater treatment plants upstream with intakes of potable water treatment plants downstream. The Rosetta River was selected as a good example of such rivers. River water is the large source of potable water as well as recreational purpose in many countries, including Egypt. Therefore, it is very important to study the presence of water-borne enteric viruses in river water to assess the risk associated with virus infection through potable water and direct/indirect contact with river water. Figure 3 summarizes the incidence of waterborne gastroenteritis viruses in Rosetta River Nile. For the 12 water and 12 sediment samples collected from Rosetta River, the detection rates in river water samples were 3/12 (25%) for AstVs, 2/12 (16.6%) for NoVs, and 1/12 (8.3%) for RVC, whereas the detection rates in sediment samples were 2/12(16.6%) for AstVs, 1/12(8.3%) for NoVs, 0/12(0%) for RVC (Figure 2).

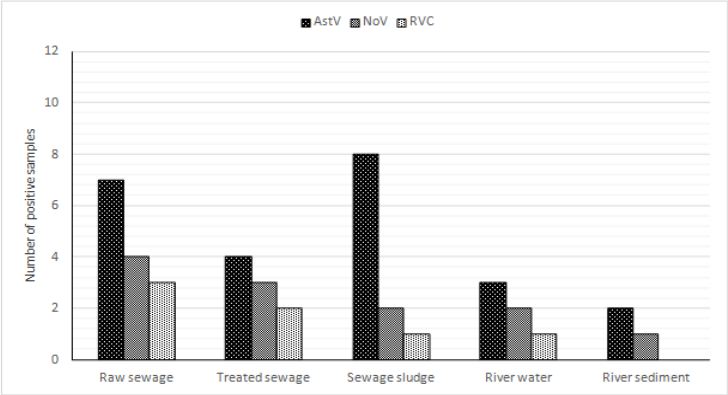

Fig. 2: Number of samples positive for astrovirus, norovirus, and group C rotavirus

The presence of AstV, NoV, and RVC in the Rosetta River water providing evidence of pollution with multiple enteric viruses. Detection rates of AstV, RVC, and NoV in river water are lower than those reported in previous studies from Egypt, in which AstV, RVC, NoV were detected in 33.3%, 12.5%, and 31.2% of Nile water, respectively (El-Senousy *et al*. 2014 and 2015). However, our results are higher than those obtained by El-Senousy *et al*. (2007) and (2014), who detected AstV (8%) and NoV (6.9%) of Nile water in Egypt (El-Senousy *et al*., 2007 and 2014). In a study from Argentina, group A rotavirus, AstV, and NoV were detected in 52.1%, 50%, and 60.4% of freshwater samples, respectively (Masachessi *et al*. 2018). Another report of AstV, NoV GI, NoV GII, and group A rotavirus incidence on Seine River in France are already described by Prevost *et al*. (2015), with 36%, 88%, 92% and 57% of positivity in water samples, respectively.

Distribution of AstVs and NoVs genogroups in the positive samples were as follows: AstV-A was detected in 2/7(28.6%) of raw sewage, 2/4 (50%) of treated sewage, 3/8(37.5%) of sewage sludge, 1/3(33.3%) of river water, and 1/2(50%) of river sediment whereas AstV-B was detected in 5/7(71.4%) of raw sewage, 2/4(50%) of treated sewage, 5/8 (62.5%) of sewage sludge, 2/3(66.6%) of river water, and 1/2(50%) of river sediment samples. On the other hand, NoV GI was detected in 3/4(75%) of raw sewage, 2/3(66.6%) of treated sewage, 2/2(100%) of sewage sludge, 1/2(50%) of river water, and 1/1(100%) of river sidemen samples whereas NoV GII was detected in 1/4(25%) of raw sewage, 1/3(33.3%) of treated sewage, 0/2 (0%) of sewage sludge, 1/2(50%) of river water, and 0/1(0%) of river sediment samples (Figure 3).

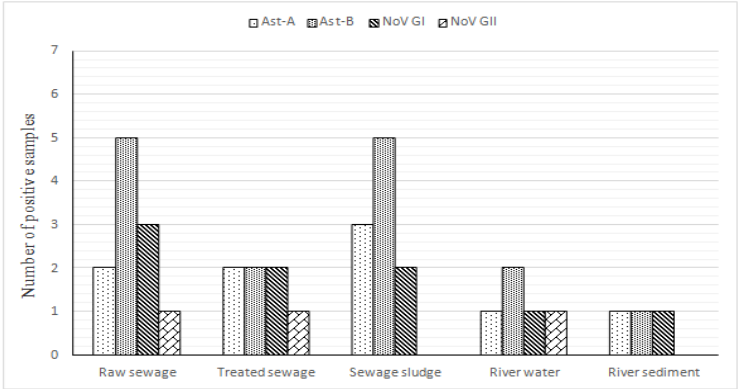

Fig. 3: Number of positive samples for astrovirus genogroups (Ast-A and AstV-B) as well as norovirus genogroups (GI and GII)

Collectively, the current study revealed a predominance of AstV-B compared to AstV-A and NoV GI compared to NoV GII in both wastewater/sewage sludge samples and water/sediment samples. These findings are similar to those documented in other studies conducted on wastewater and clinical samples in Egypt (Kamel *et al*. 2010; El-Senousy *et al.* 2014). On the contrary, previous studies from Egypt revealed a dominance of AstV-A compared to AstV-B and NoVs GII compared to NoVs GI in clinical samples (Naflcy *et al*. 2000; Kamel *et al*. 2009, and Ahmed 2011). However, high detection rates of NoVs GI in wastewater samples has been reported in several countries (Da Silva *et al*. 2007; Nordgren *et al*. 2009; La Rosa *et al*. 2010; Kittigul *et al*. 2012).

The higher frequency of AstV in sewage sludge and river sediment samples than wastewater and river water samples might be due to the presence of high amount of this virus in the sludge and sediment samples than wastewater and water samples (Prado and Fumian, 2003). In contrast, the incidence of NoV and RVC was higher in wastewater and river water samples than sewage sludge and river sediment samples. In a previous study from Brazil, adenovirus was detected in sewage sludge with higher detection rate than wastewater (Schlindwein *et al*. 2010). However, in the same study, the detection rates of hepatitis A virus and rotavirus were higher in wastewater than sewage sludge samples. The adsorption mechanisms of virus to sludge and sediment is not completely understood and may vary depending on the type of virus (da Silva *et al.* 2007) and other studies are needed to well understand this issue.

Although the current study is only one-year monitoring of these viruses, we found statistically significant seasonal variation ($p \leq 0.01$) in the incidence of AstV and RVC in raw sewage. The prevalence was higher (71.4%) for AstVs and (100%) for RVC in the cold season (October–March) than in the summer (28.6%) for AstVs and (0%) for RVC (April–September) as seen in Figure 4. The incidence of NoV did not vary significantly between the winters and summer periods.

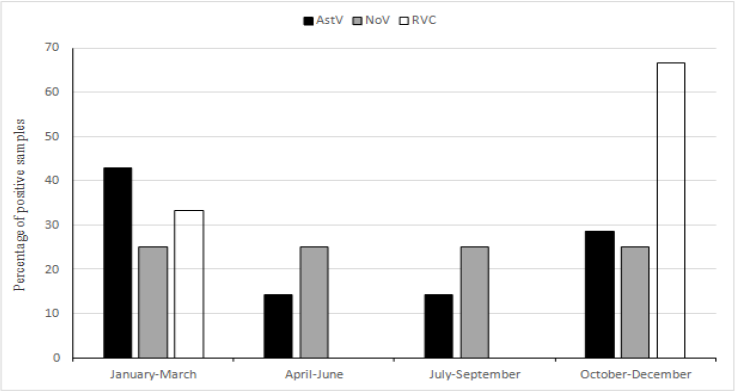

Fig. 4: Seasonal distribution of astrovirus, norovirus, and group C rotaviruses in raw sewage samples

The seasonal variations of AstV, NoV, and RVC in river water were similar to those in raw sewage (Figure 5). Our results are similar to those from other studies (Deng *et al*. 2009, Zhou *et al*. 2014; Shaheen *et al*. 2017), showed that AstV and RVs seemed to be more frequent in the winter season, while the seasonal prevalence of NoV was unclear.

Based on the present data, we can hypothesize that the direct discharge of treated effluent into the Rosetta River Nile via El-Rahawy drain affects on the water quality this river. More research, including phylogenetic analysis, needs to be

undertaken to compare the strains detected in wastewater and river water and to identify if the strain of river water was driven from wastewater.

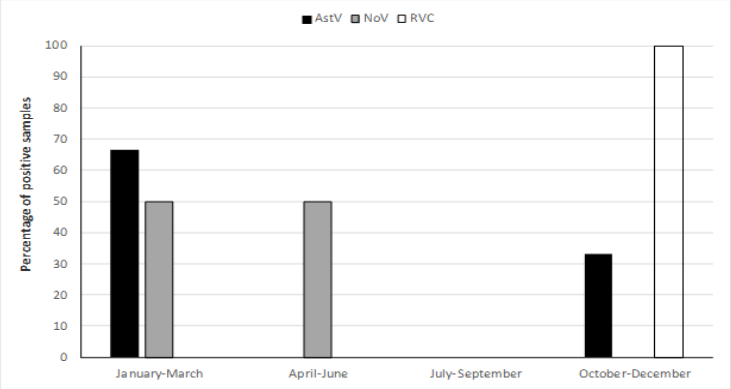

Fig. 5: Seasonal distribution of astrovirus, norovirus, and group C rotaviruses in river water samples

A long-term surveillance for monitoring these viruses in the inhabitants are highly recommended to control and/or prevent infection by these viruses. The data obtained in the current study are limited because clinical data is not found. However, when these viruses are found in sewage, it is expected that there are several individuals infected in the population and that these viruses are circulating among the community. A previous studies from Egypt conducted on clinical samples reported that NoV GI and Ast-B were the most prevalent genotypes among children with acute gastroenteritis (Kamel *et al*. 2010; El-Senousy *et al.* 2014), thus a possible correlation between the clinical and environmental genotypes may be found indicating the effect of environmental pollution on human health.

Among a large number of previous environmental and clinical studies on rotavirus in Egypt, only one study is available on group C rotavirus (El-Senousy *et al*. 2015) which detected RVC in 8.3 %, 0%, and 12.5 % of raw sewage, treated sewage, and Nile water, respectively. In addition, there are no epidemiological studies on the prevalence of AstV, NoV, and RV infections among asymptomatic persons. Monitoring of raw sewage at a WWTP could be a useful tool to understand the real prevalence of virus infections in the surrounding region of the WWTP, especially because the raw sewage contains viruses released by both symptomatic and asymptomatic cases of acute diarrhea in the surrounding area (Iwai *et al*. 2009). In addition, analyzing wastewater for enteric viruses allow us to predict outbreaks of viral gastroenteritis before it happens. As shown in previous studies, NoV and AstV can be excreted in the feces of the infected individual for several days prior to the onset of symptoms (Lee *et al*. 2013).

CONCLUSION

Our data demonstrate the significance of the investigation of wastewater and water samples to explore the actual incidence of AstVs, NoVs, and RVC among human populations. HAstV genogroup B and NoV genogroup I are the most frequent genotypes in the Egyptian environment. The current data also confirmed that the treated effluent of the WWTP can be a source of river water contamination with enteric pathogens. An efficient treatment method for removal of these viruses would contribute to reduce these waterborne pathogens and infections associated with the polluted surface water sources. In addition, future studies such as a two-year long

surveillance on both environmental and clinical samples with phylogenetic analysis are needed for better understanding of the epidemiology of AstVs, NoVs, and RVs.

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