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Molecular Detection and Genotyping of Group A Rotavirus by Multiplex Semi-Nested RT-PCR in Sewage Water and Sludge

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Research Article

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Abstract

Group A rotavirus (RV-A) infections are a major cause of morbidity and mortality in human around the world. The aim of this research was to conduct a molecular characterization of RV-A glycoprotein (G) and protease-sensitive (P) in urban sewage (n=54; 27 raw sewage and 27 treated sewage) and sewage sludge (n=27) from Egypt by multiplex semi-nested RT-PCR method. RV-A was detected in 29.9% of raw sewage, 7.4% of treated sewage, and 18.5% of sewage sludge samples. In the positive samples, the RV-A G genotypes were as follow: G3 (n=6), G9 (n=3), G1 (n=2), G4 (n=1), G10 (n=1), G1+G3 (n =1), and G1+G3+G10 (n=1) whereas P types detected included: P[4] (n=1), P[6] (n=3), P[8] (n=8), and nontypeable P (n=3). The most detected G-P combination was G3P [8] (n=6). Other G-P combinations such as G1P [8] (n=2), G9P [6] (n=2), G4P [6] (n=1), and G10P[4] (n=1) were also detected. The highest detection rates of RV-A in sewage samples were found in winter (50%), followed by summer (25%), then in spring and autumn (12.5%). The results showed not only that sewage analysis contains dramatic information concerning enteric viruses, but also that environmental monitoring is an important approach in describing the local circulation of specific viruses among population.

Keywords: Rotavirus; Sewage; Sludge; PCR

Introduction

Group A rotavirus (RV-A) is the major pathogen of sever gastroenteritis in both infants and young children in developing and developed countries, causing about 30–50 % of these diseases, with a high rates of morbidity and mortality in the population of developing countries [1]. Although, morbidity and mortality due to RV infections has reduced due to actual universal mass vaccination program against this virus, it still causes about 192,700 deaths per year in children under 5 years old [2].

RV-A, belonging to Rotavirus genus and Reoviridae family, are segmented and non-enveloped double-stranded RNA viruses. Based on the differences in the nucleotide sequences of the two outer capsid proteins (VP4 and VP7 genes) of RV-A, it has been subdivided into 27 glycoprotein (G) and 23 protease-sensitive (P)-genotypes [1,3]. RV-A strains bearing the combinations such as G1P[8], G9P[8], and G2P[4] are considered as the most prevalent genotypes in humans in the Middle Eastern and North African, accounting for 37.7%, 22.5%, and 8.1% of all RV positive samples, respectively [4].

Currently, RV-A monitoring was mostly performed in clinical cases. However, asymptomatic infection of RV-A is common [5], and case-based monitoring can only focus on the symptomatic patients. The epidemiology of RV-A in asymptomatic persons was not well studied. Because the particles of RV-A can be released from both asymptomatic and symptomatic persons to sewerage system, we can get more comprehensive data on the molecular characterizations of RV-A by sewage analysis [6]. The objective of this study was to investigate the prevalence of VP4 and VP7 genotypes of human rotavirus in sewage water and sewage sludge using semi-nested multiplex RT-PCR.

Materials and Methods

Sample Collection and Concentration

Twenty seven raw sewage, 27 treated sewage, and 27 sludge samples were collected monthly, from June 2015 to August 2017, from Zenin wastewater treatment plat (WWTP). The sewage samples were concentrated by the adsorption–elution technique, using an electronegative membrane (0.45µm pore size, and 142mm diameter), and organic flocculation method [7,8] whereas the sewage sludge samples were concentered by method described previously by EPA (1984) [9].

RNA Extraction and cDNA Synthesis

Total RNA was extracted from 1ml aliquots of the concentrated sewage and sludge samples with QIAamp Viral RNA Mini Kit (Qiagen, Germany). RT of the viral RNA was conducted using a reverse primer VP7-R (50µg/µl) and Con 3 (50µg/µl) for G and P types, respectively. Initially, 5µl of the viral RNA with 1µl of the reverse primer was heated at 65°C for 10 minutes in Biorad PCR machine (USA), then immediately snap chilled on ice. Next, 4 µl of 10 mM dNTP (Bioline, USA), 0.5 µl of Maloney murine leukemia virus reverse transcriptase **(**MMLV, Promega), 10µl of 5x RT buffer, 0.5µl RNase inhibitor, 4 µl DEPC-treated water. The mixture was incubated at 30°C for 1 hr, 42°C for 30 min, then 95°C for 5 min at the end of the incubation step.

G and P Genotyping

Amplifying of a VP7 gene was performed using two steps according to protocol described previously by Iturriza Gomara et al. (2004) [10]. The first-round of PCR amplification of the VP7 gene was performed with 5 of cDNA, 10 µl of M-MLV 5X reaction buffer, 4 µl of 25 mM $MgCl_2$, 4 µl of 10 mM dNTPs, 0.25 µl of 5 U/ ml Go Taq DNA polymerase (Promega, USA) and 1 µl of 25 pmol of each primer (Table 1), 24.75 µl DEPC-treated water. PCR conditions on the thermocycler (Bio-Rad, Singapore) were as follows: 95°C (5 min); followed by 35 cycles consisted of 95°C (1 min), 52°C (1 min), and 72°C (1 min); then a final extension step at 72°C (10 min). The second round VP7 multiplex was performed in 50 µl total volume containing a 2.5 µl of the first PCR product as a template with 1 µl of each G-type-specific primer or P-type-specific primer (Table 1), 10 µl of M-MLV 5X reaction buffer, 4 µl of 25 mM $MgCl_2$, 4 µl of 10 mM dNTPs, 0.25 µl of 5 U/ml Go Taq DNA polymerase (Promega, USA), 23 µl DEPC-treated water. The thermal cycling conditions were as follows: 95°C (5 min); followed by 30 cycles at 94°C (1 min), 42°C (2 min), 72°C (1 min); then a final extension step at 72°C (10 m).

Primer		Sequence (5-3)	Amplicon (bp)	nt. Position	Ref.
G-typing					
1st round	VP7-F	ATG TAT GGT ATT GAA TAT ACC AC	881	51-71	[11]
	VP7-R	AAC TTG CCA CCA TTT TTT CC		914-932	
2nd round	G3	ACG AAC TCA ACA CGA GAG G	682	250-269	[10,12]
	G9	CTT GAT GTG ACT AY[a] A AAT AC	179	757-776	
	G10	ATG TCA GAC TAC AR[b] A TAC TGG	266	666-687	
	G8	GTC ACA CCA TTT GTA AAT TCG	754	178-198	
	G4	CGT TTC TGG TGA GGA GTT G	452	480-499	
	G2	CAA TGA TAT TAA CAC ATT TTC TGT G	521	411-435	

	G1	CAA GTA CTC AAA TCA ATG ATG G	618	314-335	
	VP7-R	As above			
P-typing					
1st round	**Con3**	TGGCTTCGCCATTTLATAGACA	876	11-32	[13]
	Con2	ATTTCGGACCAT'lTATAACC		868-887	
2nd round	**P[8]**	TCTACTTGGRTTRACNTGC	345	339-356	[10,13, 14]
	P[4]	CTATTGTTAGAGGTTAGAGTC	483	474-494	
	P[6]	TGTTGATTAGTTGGATTCAA	267	259-278	
	P[9]	TGAGACATGCAATTGGAC	391	385-402	
	P[10]	ATCATAGTTAGTAGTCGG	583	575-594	
	P[11]	GTAAACATCCAGAATGTG	312	305-323	
	Con3	As above			

Ya = C or T; Rb = A or G; N= A, G, C or T

Table 1: Primers used for detection and G and P typing of RV

Results

Prevalence and Seasonal Variation of Group A Rotavirus

RV-A was detected in 29.9% (8/27) of raw sewage, 7.4% (2/27) of treated sewage, and 18.5% (5/27) of sewage sludge. The highest detection rate of rotavirus in raw sewage was found in winter season (5/8, 62.5%), from December to February. In the other seasons, the detection rate of RV-A was 25% (2/8) in summer season (June-August) whereas it was 12.5% (1/8) in autumn (September-November) and 0% in spring (March-April). In treated sewage samples, the detection rates of RV-A was 50% (1/2) in winter 2016, 50% (1/2) in summer 2017 whereas no virus was detected in spring and autumn. In sewage sludge, RV-A was found in 40% (2/5) of autumn, 40% (2/5) summer, 20% (1/5) of winter, and 0% (0/4) (Figure 1).

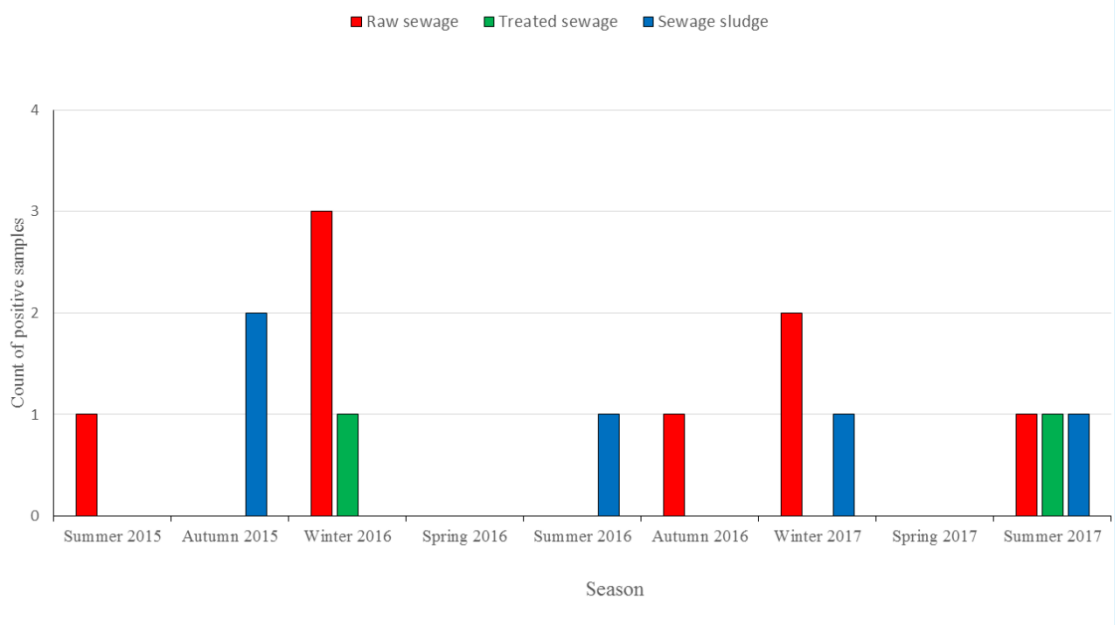

Figure 1: Semi-nested RT-PCR results for the detection of rotaviruses in raw sewage, treated sewage, and sewage sludge samples.

Molecular Characterization of the Detected RV

Analysis VP7 gene by multiplex semi-nested RT-PCR showed that G3 (6/15, 40%) was the most genotype strain, followed by G9 (3/15, 20%), and G1 (2/15, 13.3%). G4 and G10 genotypes were identified in the same percentage (1/15, 6.6%) of strains. Two mixed infections with two (G1+G3; 1/15, 6.6%) or three (G1+G3+G10; 1/15, 6.6%) G genotypes were also detected. Analysis VP4 gene showed that P[8] is the most common strain (53.3%; 8/15), followed by P[6] 20%; 3/15, and P[4] 6.6%; 1/15. P[9], P[10], and P[11] strains were not detected in this study. Some positive samples (20%; 3/15) were non-typeable for G-P combination (Figure 2). The most common genotype combination was G3P[8] (6/15; 40%). G1P[8] and G9P[6] genotype combinations were detected in the same percentage 13.3%, 2/15. Other genotype combinations such as G4P[6] and G10P[4] were also detected in this study (6.6%, 1/15). The genotype combinations were not detected in some rotavirus-positive environmental samples (20%, 3/15) (Figure 3).

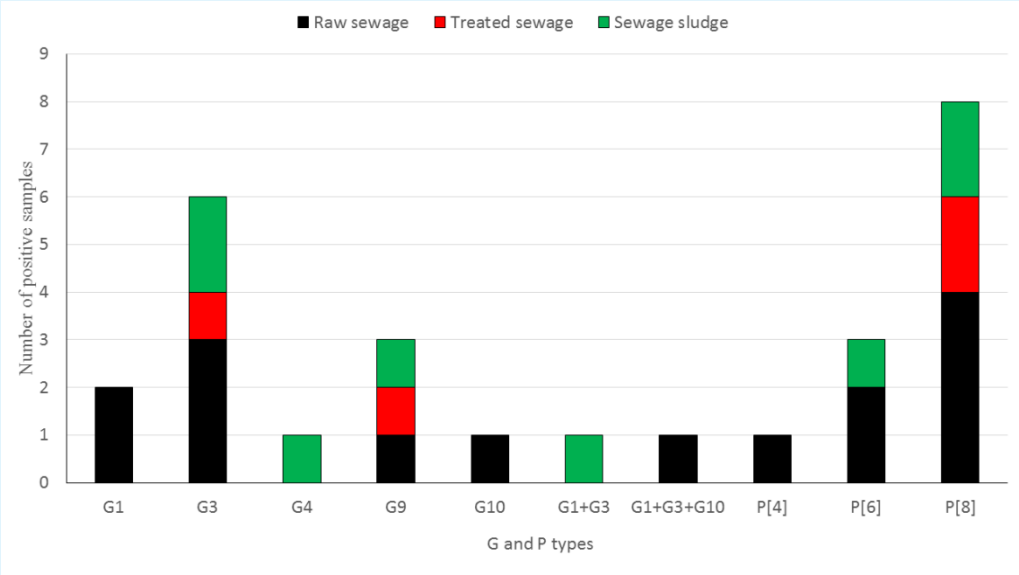

Figure 2: Rates of identification of RVA G types and P types detected in raw sewage, treated sewage, and sewage sludge from Egypt

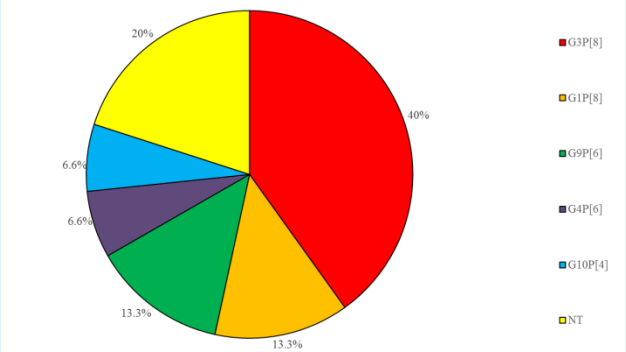

Figure 3: Rates of identification of RVA G-P combination detected in raw sewage, treated sewage, and sewage sludge from Egypt

Discussion

Environmental sewage monitoring has been successfully performed to get data on molecular characterizations of various enteric viruses in Egypt [15-18]. In this research, we investigated the prevalence, genotypes, and seasonality of RV-A in Giza government, Egypt. During the 27-month period, a total of 27 raw sewage, 27 treated sewage, 27 sewage sludge samples were collected monthly. In order to improve the sensitivity of detection method, we applied semi-nested PCR for the collected samples in this study. The high sensitivity of the nested RT-PCR detection has been previously described for double stranded RNA RV-A that could detect a few viral particles (10-100 particles) [13].

After conducting of semi-nested multiplex RT-PCR detection, rotavirus was identified in 29.9% (8/27), 7.4% (2/27), and 18.5% (5/27) of influent, effluent, and sewage sludge, respectively. Although the number of RV-A positive specimens was decreased upon treatment in the current wastewater treatment plant, the treated effluent still contained RV-A nucleic acids. This finding highlights the high risk of RV-A infection among the population when this effluent is reused in agricultural production and/or its discharge in bathing and recreational water.

In two previous studies conducted on the same wastewater treatment, RV-A was detected in 5/12(41.6%) and 16.7% of raw sewage and in 0% and 16.7 of treated sewage during 2006-2007 and 2009-2011, respectively [18,19]. Moreover, the current viral prevalence is relatively high when compared with other previous reports. Differences in the prevalence of RV-A might be due to the variations in sensitivity of virus recovery and detection methods. For example, two reports from Bangkok documented that 8% and 0% of untreated sewage specimens were positive for rotavirus by ELISA technique [20,21]; while by using indirect immunofluorescence, 21% of the samples were positive for rotavirus in a study from Sao Paulo [22]. Moreover, although a semi nested RT-PCR was applied in a study conducted in Barcelona, rotavirus was identified in only 4 of 15 sewage samples [23]. A similar study from France, however, documented a prevalence of rotavirus in 42% of untreated sewage [24,25], which is higher than our current results. Two studies from Tunisia and China, found RV in 72·4% and 93.5% of sewage samples using real time PCR, respectively [6,26]. Moreover, our detection rates of RV in sewage sludge is lower than those detected in study from Brazil [26].

Group A rotavirus should be recognized as potential marker of fecal pollution present in the aquatic environment since RV-A movement appears to be greater than the adenovirus, which is recognized as a potential indicator of the occurrence of enteric viruses in the environment [27]. RV-A stability in water environment, and its resistance to physico-chemical processes applied for sewage treatment promotes its dissemination and transmission in the water environment [28].

Genotyping was conducted on fifteen rotavirus positive sewage and sludge samples by multiples semi-nested RT-PCR. The most prevalent genotypes were G3, G9, P[8], P[6], with G3 P[8] being the most prevalent genotype combination in the positive samples. El-Senousy et al, (2014) [16] stated that G3 and P[8] were the second most common genotype after G1 and P[4] in water samples from Egypt. In agreement with our finding, G9 was the second most common genotype in sewage samples [29]. Indeed, G1 and P[8] were documented as the most common genotypes in Spain and Brazil [28,30] as well as G1 and P[8] in Venezuela [31]. In agreement with our findings, genotype G3P[8] was found as the most frequent genotype in wastewater samples from Tunisia [32]. However, G1P[8] combination was documented as the most common genotype in the clinical and environmental samples from several countries, including the Middle Eastern and North African Region [4,28,30,33].

This study is limited due to absence of clinical data. However, our previous studies demonstrated that genotypes G3 [34] and P[8] (unpublished data) were the most predominant genotypes in clinical samples from children with severe diarrhea which agree with this study. Thus, G3 and P[8] detected in the population might be originated from contaminated sewage. Sewage monitoring system has been observed to be more accurate than reporting of clinical cases of serious diseases in a population [35]. Data from the presence of enteric viruses in untreated sewage may provide a useful data on the epidemiology of enteric virus infections circulating in the population, including asymptomatic infections [36,37].

The present research showed a higher detection rates of RV-A during the winter season. The same seasonality pattern has been reported in our previous study on clinical surveillances [34]. Seasonal occurrence of group A rotavirus in sewage with a prevalence peak in winter has been stated in other studies [6,15,38,39]. The predominance of viral infections during the winter months, which refer to the possible transmission of viral gastroenteritis via a respiratory route, still not fully understood. However, increased virus stability in the environment at the lowered temperature was found in reports on astrovirus, poliovirus, and HAV [40,41], could contribute to waterborne viruses during winter season, and therefore a higher viral concentrations in sewage.

The resistance of group A rotavirus to the active sludge treatment process agrees with previous reports that also found human sapovirus, astrovirus, and norovirus, in influent and effluent samples by using different types of sewage treatment, including chlorination treatment [42-46]. Although RT-PCR-based method is a fast and accurate tool for the detection of group A rotavirus, it does not differentiate between viable and non-viable virus. However, the presence of viral

nucleic acids in water specimens can be of significant value as a marker of recent viral pollution because of the low stability of free genomes in water environments, especially RNAs [47].

Conclusion

Our study has characterized VP7 and VP4 genes of group A rotavirus from sewage samples demonstrating that G3 and P[8] strains are the most prevalent in the environment and therefore this study suggests that environmental surveillance of sewage provides a good assessment of group A rotavirus genotypes circulating in the local human community. Future studies should include both clinical and environmental samples and investigate the occurrence of additional enterically transmitted viruses to obtain powerful data for both epidemiological purposes and outbreak early warning.

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