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Molecular characterization of rotavirus Group A VP6 gene in Egyptian surface water, wastewater and diarrheal specimens

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ABSTRACT

Rotaviruses are the major cause of viral gastroenteritis in infants and young children, producing a significant pediatric disease burden worldwide. The objective of this study was to investigate mutations in the group A human rotaviruses (RoV) in Egypt and their VP6 gene in Egyptian clinical specimens, raw sewage, treated effluents, Nile water, and drinking water samples. A total of 1026 diarrheal specimens were collected from Abo EL-Reesh children hospital between October 2015 and September 2017. Human RoV group A was detected in 22.61% of the clinical specimens. The highest peak of RoV was noticed in autumn and winter. The detection rate of RoV in sewage samples was 88.6% and 65.7% for the influent and effluent samples respectively. Additionally, RoV was detected in 64.29% of river water samples and in 42.86% of drinking water samples. Sequence analysis of the full-length VP6 gene of clinical and environmental samples revealed silent and non-silent mutations compared to RoV Wa reference strain. Different sequences were clearly clustered with genotypes G1P8, G4P6 and G2P4. The low variations between sequences of the VP6 gene in both clinical specimens and environmental samples which contained the same human RoV genotype and also the high similarity between the VP6 genes in samples which contained different human RoV genotypes support the idea of using VP6 as a candidate human RoV recombinant subunit vaccine in Egypt.

INTRODUCTION

One of the leading infectious disease causing morbidity and mortality in children under 5 years is diarrheal diseases (GBD, 2016), moderate-to-severe diarrhea in young children, is well associated with RoV infection as the major infectious agent behind it

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(Kotloff *et al.*, 2013; Liu *et al*., 2016). The global burden of RoV acute gastroenteritis (AGE) hospitalization and deaths are well documented. RoV AGE was responsible for 30–50% of AGE hospitalizations in both developing and developed countries in the pre vaccine era (CDC, 2007; Mwenda *et al*., 2015; CDC, 2016; Burnett *et al*., 2017). RoV mortality disproportionately affects developing countries, with about 82–95% of mortalities among children under 5 years of age occurring in these areas (Burnett *et al*., 2017). Annually, about 829 000 individuals are estimated to die because of diarrhea as a result of unsafe drinking-water, sanitation, and hand hygiene. Nonetheless, diarrhea is basically preventable, and the deaths of 297, 000 children aged beneath 5 years could be avoided every year if these risk factors were addressed (WHO, 2019).

All of the RoV outbreaks have been associated with direct fecal contamination of untreated, compromised water supply or suboptimal treatment of drinking water. RoV have been detected in surface waters worldwide (Gerba, 1999; Tort *et al*., 2015; Yousuf *et al*., 2017). Surface water receiving untreated wastewater effluent; have been reported to contain the highest concentrations of viral pathogens (Griffin *et al*., 2003). In natural disasters, fecal matter and potable waters can mix, permitting infective concentrations of RoV to exist in water supplies, causing a risk for the public health (Gutiérrez-Aguirre *et al*., 2008).

RoVs are excreted in monumental numbers in the stool, described to be more than 10^{11} particles per gram (Flewett, 1982) and are transmitted mainly via fecal-oral route. RoVs are incredibly stable in stool and can stay viable at room temperature for days, making them extremely resilient and easily transmitted in several settings, including hospital wards, maternity units, and day care centers for young children (Flewett, 1982).

In 2006, two oral live attenuated RoV vaccines were licensed for infants up to 6 months of age, a monovalent human RoV vaccine (RV1, Rotarix, Glaxo Smith Kline Biologicals, Rix-ensart, Belgium) and a pentavalent bovine-human reassortant vaccine (RV5, Rota Teq, Merck Vaccines, White house Station, NJ,USA). Efficacy against the serotypes included in the two vaccine had been proven through clinical trials (Ruiz-Palacios *et al*., 2006; Vesikari *et al*., 2006). In February 2006, RoV vaccination was recommended for the first time to children in the United States (Wang *et al.*, 2010). Subsequently, in April 2009, WHO recommended that RoV vaccine be included in every country's national immunization program (WHO, 2009). Since 2006, 109 countries around the world had introduced (includes partial introduction) RoV vaccines into their pediatric immunization programs (WHO, 2020). Many of these countries had since documented substantial declines in RoV disease burden in both vaccinated and unvaccinated children (Melliez *et al*., 2007; Tate *et al*., 2009; Wang *et al*., 2010; Paulke-Korinek *et al*., 2010; do Carmo *et al*., 2011; Lanzieri *et al*., 2011; Bayard *et al*., 2012).

According to Snelling *et al*., 2011, there is no evidence that RoV vaccine can offer a protective effect against outbreaks of heterotypic strains. However, in evaluations of these vaccines in the population, most studies, had found them to be highly effective under real life conditions (Castilla *et al*., 2012;Yeung *et al*., 2016; Abebe *et al*., 2018; Pietsch & Liebert, 2019;). Despite being highly effective, future vaccination in low-income, high-burden countries in Africa and Asia may have a large financial implication. In addition, these countries also showed lower vaccine efficacy (51–64%) than in high- and middle-income countries (Bresee *et al*., 2005; Armah *et al*., 2010; Tate *et al*., 2010; Stockman, 2011; Tate *et al*., 2012).

There are six main RoV genotype combinations; G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] which have been associated worldwide with the majority of RoV gastroenteritis infections in humans (Matthijnssens and Van Ranst, 2012). Although the distribution of these six globally important RoV genotypes can change drastically from region to another and from one year to the next, the most prevalent RoV strain worldwide has remained G1P[8] strain (Rahman *et al*., 2005; Santos and Hoshino, 2005; Van Damme *et al*., 2007). However, significant diversity of RoV genotypes continues to be observed worldwide with several novel combinations due to accumulation of point mutations, genome reassortment, and/or zoonotic transmission to human host resulting in the introduction of new antigenic variants across regions (Matthijnssens *et al*., 2009; Martella *et al*., 2010). G1P8 was the most prevalent genotype in Egypt (Villena *et al*., 2003; El-Senousy *et al*., 2004) with the tendency of increasing the prevalence of G1P4 genotype in the last years to be considered as the higher prevalent genotype in the Egyptian clinical and environmental samples in parallel to G1P8 (El-Senousy *et al*., 2014a; El-Senousy and Abou-Elela, 2017).

VP6 protein is the sole structural protein of the middle of three capsid layers of RoV. It has a theoretical molecular mass of 45 KD. It is the most conserved, immunogenic, and abundant RoV protein, a single human RoV VP6 protein may elicit protection against all human RoV strains belonging to multiple serotypes (Choi *et al*., 2004). VP6 protein carries antigen determinants that are common to all group A RoV; it also contains epitopes that have been used to classify the group A viruses into subgroups (Lopez-Guerrero *et al*., 2018). The major capsid protein VP6 is an immune dominant antigen with high sequence homology and common antigenic epitopes among group A RoV (Estes & Cohen, 1989). In this context we aimed to estimate the silent and non-silent mutations in RoV VP6 gene in order to know the degree of sequence diversity of the human ROV circulating strains in Egypt.

MATERIALS AND METHODS

Clinical specimens

A total of 1026 stool samples were collected during the period from October 2015 to September 2017 from Abo El-Reesh children hospitals in Greater Cairo from children (< 5 years old) suffering from acute diarrhea. Samples were collected in clean containers and transferred to the laboratory within 2 hrs of collection for examination. Approximately 0.1 g of stool samples was weighed, diluted 1:10 in nuclease-free H_2O, and vortexed for 30 sec. Samples were clarified by centrifugation at 7.000 rpm for 10 min at room temperature. Viral RNA was extracted from 100 µl of the supernatant.

Sewage and water samples

A total of 28 samples were collected from October 2015 to February 2018 from Giza drinking water treatment plant (WTP), 20 liters of water samples were collected including inlet (Nile water), and outlet water (drinking water). Sodium thiosulfate (BDH Chemicals Ltd Poole England) was added to the chlorinated samples to a final concentration of 5 mg/l, to inactivate chlorine. Aluminum chloride (1ml/l) was also added to increase the stability of the viruses in the samples during transportation (APHA, 1998).

During the same period, a total of 28 sewage samples were collected from Zenin wastewater treatment plant (WWTP-1) which works by activated sludge system, with

flow total capacity of 330,000 m^3/day. Also, 42 sewage samples were collected in clean bottles from both the influents and chlorinated effluents of EL-Gabal EL-Asfer wastewater treatment plant (WWTP-2) and transported to the laboratory within 3 hrs of collection for examination. Sodium thiosulfate (BDH Chemicals Ltd Poole England) was added to the chlorinated samples to a final concentration of 5 mg/l to inactivate chlorine. Viruses were concentrated from one to three liters of water or wastewater samples by the aluminum hydroxide adsorption-precipitation method (APHA, 2017).

Viral nucleic acid extraction

Viral nucleic acid was extracted using BIOZOL Total RNA Extraction reagent (BIOFLUX, Japan) and according to the manufacturer's instructions. Standard Precautions were followed to avoid contamination. Negative control sample (sterile nuclease-free water) was included in each extraction session to monitor cross-contamination.

Molecular detection of group A Rotavirus VP6 gene in clinical specimens

For generic detection of RoV, a reverse transcription (RT)-PCR–hybridization method based on amplification of a VP6 fragment and confirmation by Southern blot hybridization with a digoxigen in-labeled internal probe was used. Primers VP6-3 (5-GCTTTAAAACGAAGTCTTCAAC-3; positions 2 to 23 of human strain Wa [accession number K02086]) and VP6-4 (5-GGTAAATTACCAATTCCTCCAG-3; positions 187 to 166 of human strain Wa [accession number K02086], each at a concentration of 1M, were used in an RT reaction in a 10µl (final volume) mixture containing 4U of Moloney murine leukemia virus enzyme (Promega), each deoxynucleoside triphosphate at a concentration of 0.2 mM, and 5µl of a denatured (5 min at 99°C) double-stranded RNA sample. The reaction mixture was incubated for 60 min at 50°C. PCR was performed by using 5µl cDNA and 3.5 U of the Expand High Fidelity PCR system (Roche) in a 50 µl mixture supplemented with each primer at a concentration of 1M and each deoxynucleoside triphosphate at a concentration of 2 mM. The PCR program included a 9-min denaturation step at 95°C and 40 cycles of amplification for 1 min at 94°C, for 1 min at 50°C, and for 1 min at 72°C, followed by a final elongation step of 7 min at 72°C (Villena *et al.,* 2003). VP6 detection in clinical positive samples was confirmed by sequencing of the amplified 186 bp fragment. These specimens were previously screened for RoV VP6 by our group (Food-Borne viruses group, NRC) (El-Senousy *et al*., 2020) using nested RT-PCR according to (Iturriza Gomara *et al*., 2002; Gallimore *et al*., 2006).

Nested RT-PCR for detection of VP6 gene in water and waste water samples

The primers used for RT-PCR were the forward VP6-F 5-GACGGNGCNACTACATGGT-3 and the reverse VP6-R 5-GTCCAATTCA TNCCTGGTGG-3 primers (1 µm for each), and according to (Iturriza Gomara *et al.,* 2002) using 200 U of M-MLV reverse transcriptase enzyme (Biobasic, Canda) in a total volume of 10 µl and 1.5 U of Taq DNA polymerase (Biobasic—Canada) in a total volume of 50 µl. Nested PCR amplification of the target rotavirus VP6 fragment was performed using the forward primer, VP6-NF 5-GCTAGAAATTTTGATACA-3, and the reverse primer, VP6-NR 5-TCTGCAGTTTGTGAATC-3 (1 µm for each), and according to Gallimore and co-workers (Gallimore *et al.,* 2006) using High-fidelity- DNA

polymerase to amplify 155 bp fragment. PCR products (10 µl) were analyzed by electrophoresis on 3% agarose gels (Panreac—Spain).

Amplification of VP6 full-length gene

The clinical specimens and environmental samples were previously tested for VP8 partial gene (El-Senousy *et al*., 2020) Fourteen samples which contained G1P8 genotype (7 clinical specimens, 3 raw sewage, 2 treated effluents, 1 raw Nile water, and 1 drinking water sample), seven samples which contained G2P4 (4 clinical specimens, 1 raw sewage, 1 treated effluents, 1 raw Nile water sample), and 5 samples which contained G4P6 genotypes (3 clinical specimens, 1 raw sewage, and 1 raw Nile water sample) were chosen for amplification of their whole VP6 gene followed by sequencing this amplified gene. The description of clinical specimens and environmental samples are shown in tables 1a and 1b respectively.

The amplification of whole sequence of VP6 gene was performed according to (Zhou *et al*., 2010), using the primers pair VP6-8 (5′-ATGGAGGTTCTGTACTC- 3′), VP6 nt 1–17 and VP6-2 (5′-TCACTTAATCAACATGC-3′), VP6 nt 1194–1178.

Table 1a. The description of clinical specimens

ID of diarrheal Specimens	Date	Gender	Age	Genotype
CS1	Nov.2015	Male	9 Months	G1P8
CS2	Nov.2015	Female	9 Months	G1P8
CS3	Jan.2016	Male	17 Months	G1P8
CS4	Jan.2016	Male	5 Months	G1P8
CS5	Oct.2016	Male	12 Months	G1P8
CS6	Nov.2016	Female	12 Months	G1P8
CS7	Dec.2016	Male	7 Months	G1P8
CS8	Nov.2015	Male	6 Months	G2P4
CS9	Jan.2016	Female	12 Months	G2P4
CS10	Jan.2016	Male	6 Months	G2P4
CS11	Jan.2017	Female	6 Months	G2P4
CS12	Dec.2015	Male	15 Months	G4P6
CS13	Dec.2016	Male	8 Months	G4P6
CS14	Jan.2017	Female	24 Months	G4P6

Table 1b. The description of and environmental samples

ID of environmental samples	Date	Type of sample	Genotype
ES1	Nov.2015	Raw sewage WWTP-21	G1P8
ES2	Dec.2015	Raw sewage WWTP-2	G1P8
ES3	Dec.2015	Treated effluent WWTP-2	G1P8
ES4	Feb.2017	Raw sewage WWTP-1	G1P8
ES5	Feb.2017	Treated effluent WWTP-1	G1P8
ES6	Dec.2016	River water	G1P8
ES7	Dec.2016	Drinking water WTP	G1P8
ES8	Mar. 2016	Raw sewage WWTP-1	G2P4
ES9	Mar. 2016	Treated effluent WWTP-1	G2P4
ES10	Jan.2017	River water	G2P4
ES11	Feb.2017	Raw sewage WWTP-2	G4P6
ES12	Feb.2017	River water	G4P6

Sequencing and Phylogenetic analysis of human Rotavirus VP6 gene in clinical specimens and environmental samples

The RT-PCR of selected positive RoV samples was purified by PCR products purification kit (Qiagen) and DNA was sequenced with an ABI prism 310 automated DNA sequencer. Sequence data from both strands of the PCR products were aligned and compared using the CLUSTALW and BLAST programs (European Bioinformatics Institute). Sequence analysis was conducted to examine the silent and non-silent mutations in the amplified whole VP6 gene plus the evolutionary relationship between the Egyptian clinical specimens and environmental samples with human RoV reference strains recorded in the GeneBank. Also, a neighbor joining tree was constructed with 1000 bootstrap replicates using Mega X software.

RESULTS

Human rotavirus VP6 in clinical specimens

Human rotavirus VP6 was detected in 22.61% (232/1026) of the clinical specimens with tendency to higher prevalence in autumn and winter months (Fig.1).

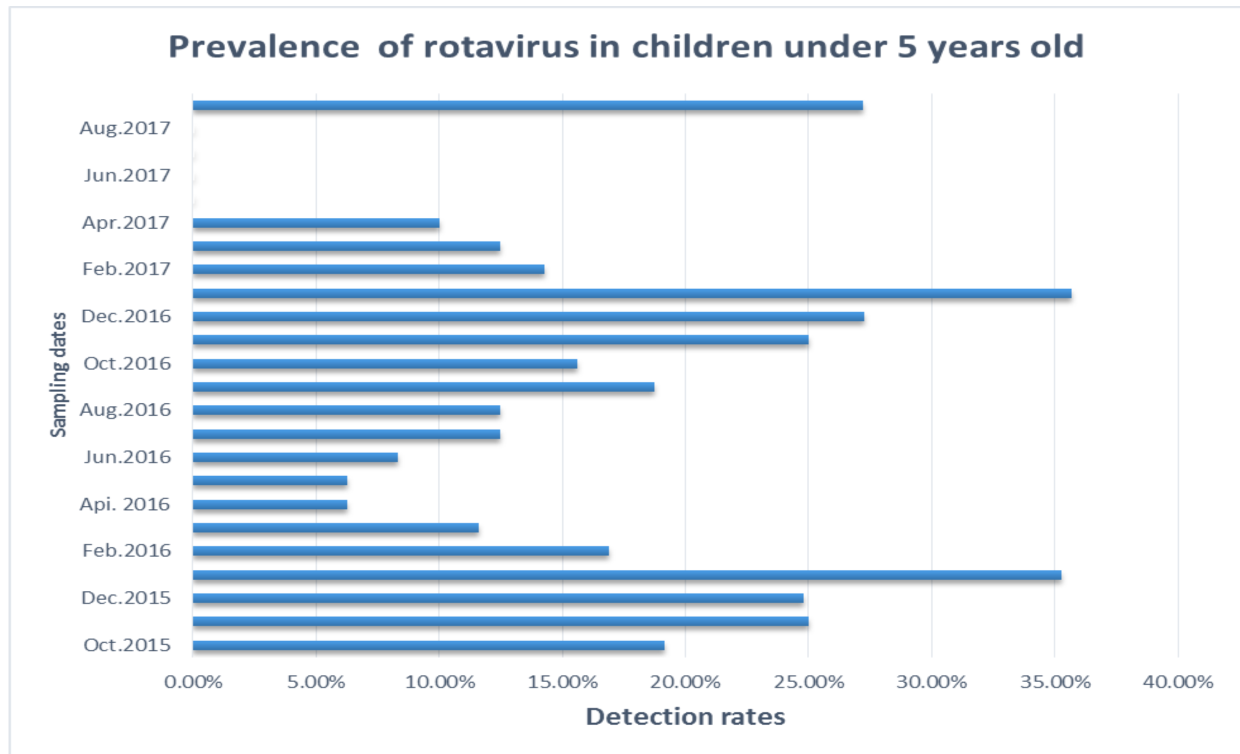

Fig. 1: The incidence of rotavirus group A in children (< 5 years old) suffering from acute diarrhea.

Human rotavirus VP6 in sewage and water samples

Human RoV VP6 was detected in 90.48% (19/21) of raw sewage samples of WWTP-2, while it was detected in 66.67% (14/21) of the treated effluents of the same WWTP-2. Also, the virus was found in 85.71% (12/14) and 64.29% (9/14) of influent and effluents of WWTP-1, respectively. On the other hand, human RoV VP6 was detected in 64.29% (9/14) of the river water samples (inlet of WTP), while it was detected in 42.86% (6/14) of the drinking water samples of the same WTP (Fig.2).

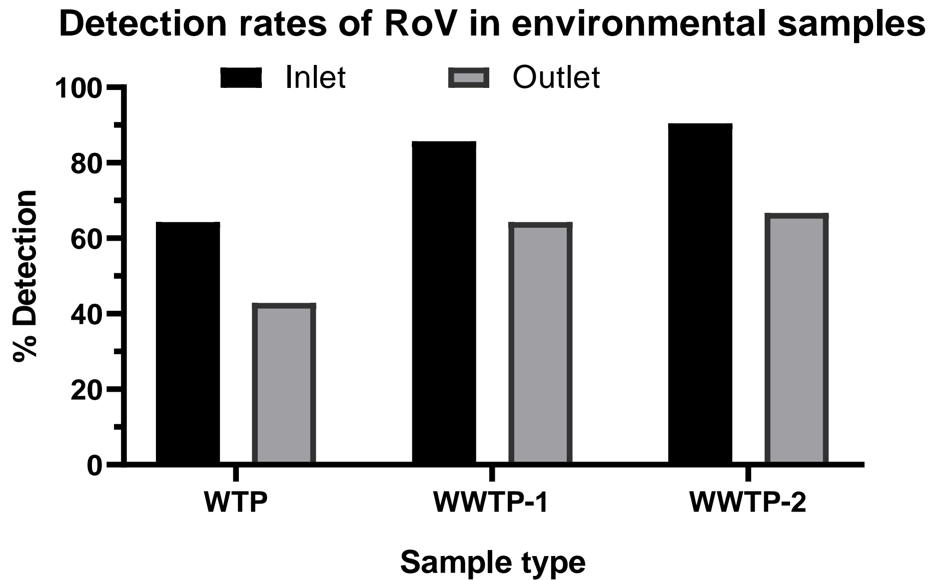

Fig. 2: The prevalence of rotavirus group A in water and wastewater samples

Sequencing of human rotavirus VP6 whole gene

Sequence analysis of human RoV VP6 whole gene of RoV isolates have been compared to some human RoV sequences that deposited in GeneBank (Fig.3). The three clinical specimens (CS2, CS3, and CS5) and 4 environmental samples (ES2, ES3, ES6, and ES7) had similar sequences and showed also 32 nucleotide substitutions including 7 non-silent mutations resulting in 7 amino acid changes (Proline changed to Arginine, Leucine changed to Phenylalanine, Leucine changed to serine, Alanine changed to serine, Threonine changed to Leucine, Glutamic acid changed to Glutamine, Leucine changed to Valine, at positions: 102, 125, 126, 241, 279 and 327, and 382, respectively). They showed 98% nucleotides identity and 98% amino acid identity in comparison to human RoV Wa reference strain, GeneBank nucleotides accession number: **K02086.1** and amino acid accession number: **P03530.1** (Table. 2).

Other 4 samples [2 clinical specimens (CS5 and CS7), 1 raw sewage samples (ES4) and 1 treated effluent sample (ES5)] had similar sequences with 25 nucleotides substitutions including 7 non-silent mutations resulting in 7 amino acid changes (Alanine changed to Glycine, Isoleucine changed to Threonine, Alanine changed to serine, Valine changed to Alanine, Threonine changed to Arginine, Phenylalanine changed to serine, Valine changed to Leucine at positions: 198,199, 241,259, 301, 308, and 346 respectively). Sequences showed 98% nucleotides similarity and 98% amino acid identity in comparison to human RoV Wa reference strain (GeneBank nucleotides accession number: **K02086.1** and GeneBank amino acid accession number: **P03530.1** (Table. 2).

One clinical specimen (CS4) showed 19 nucleotide substitutions including 1 non-silent mutations resulting in 1 amino acid change (Alanine changed to serine at position 241) with 98.56% nucleotides identity and 99.75% amino acid identity to human RoV Wa reference strain.

The clinical specimen (CS1), and environmental sample (ES1)] had similar sequences and showed 2 nucleotide substitutions including 1 non-silent mutations

resulting in 1 amino acid change (Alanine changed to serine at position 241) with 99.85% nucleotides identity and 99.75% amino acid identity to human RoV Wa reference strain.

On the other hand, 4 clinical specimen (CS8, CS9, CS10 and CS11) and 3 environmental samples (ES8, ES9, and ES10) had similar sequences and showed highest relation to G2P4 human RoV strain (GeneBank nucleotides accession number: **KJ721705.1** and GeneBank amino acid accession number: **AET43486.1**) with 93.95% amino acid identity to human RoV Wa reference strain (Fig.3).

Also, 3 clinical specimen (CS12, CS13, and CS14) and 2 environmental samples (ES11 and ES12) had similar sequences and showed highest relation to G4P6 human RoV strain (GeneBank nucleotides accession number: **JN129111.1** and GeneBank amino acid accession number: **AFK27517.1**) with 88.86 % nucleotides identity and 96.98% amino acid identity to human RoV Wa reference strain (Table. 2).

The 4 groups of sequences of samples (clinical and environmental) which contained G1P8 genotype had nucleotide similarity ranged from 97.42% to 98.26 % and amino acid similarity ranged from 96.98% to 98.49 % as well between each others (Fig.3). On the other hand, the amino acid similarity ranged from 92.19% to 93.70% between the sequence of samples which contained G2P4 genotype and the sequences of samples which contained G1P8 genotype. Also, the amino acid similarity ranged from 95.21 % to 96.73% between the sequence of samples which contained G4P6 genotype and the sequences of samples which contained G1P8 genotype. The amino acid similarity between sequence of samples which contained G2P4 genotype and sequence of samples which contained G4P6 genotype was 93.45%.(Table. 2).

Table 2. Comparison the similarities of nucleotide sequences and Amino acid identity sequences between Egyptian rotavirus A VP6 gene (from Egyptian clinical specimens and environmental samples) and RoV A reference strains (rotavirus A strain/Wa and rotavirus A strain/USA).

Strain name	**Nucleotide accession**	**Protein accession**	**Nucleotide identity with the Egyptian sequences (%)**					**Amino acid identity with the Egyptian sequences**				
			CS2 CS3 CS5 CS6 CS7 ES2 ES3 ES5 ES6 ES7	**CS4**	**CS1 ES1**	**CS8 CS9 CS10 CS11 ES8 ES9 ES10**	**CS12 CS13 CS14 ES11 ES12**	**CS2 CS3 CS5 CS6 CS7 ES2 ES3 ES5 ES6 ES7**	**CS4**	**CS1 ES1**	**CS8 CS9 CS10 CS11 ES8 ES9 ES10**	**CS12 CS13 CS14 ES11 ES12**
Rotavirus A strain/Wa	**K02086.1**	**P03530.1**	**98**	**98.56**	**99.85**	**0**	**88.86**	**98**	**99.75**	**99.75**	**93.95**	**96.98**
Rotavirus A strain USA	**KCS79987.1**	**AGE99045 .1**	**98**	**98**	**100**	**0**	**88.78**	**98**	**100**	**100**	**93.70**	**96.73**

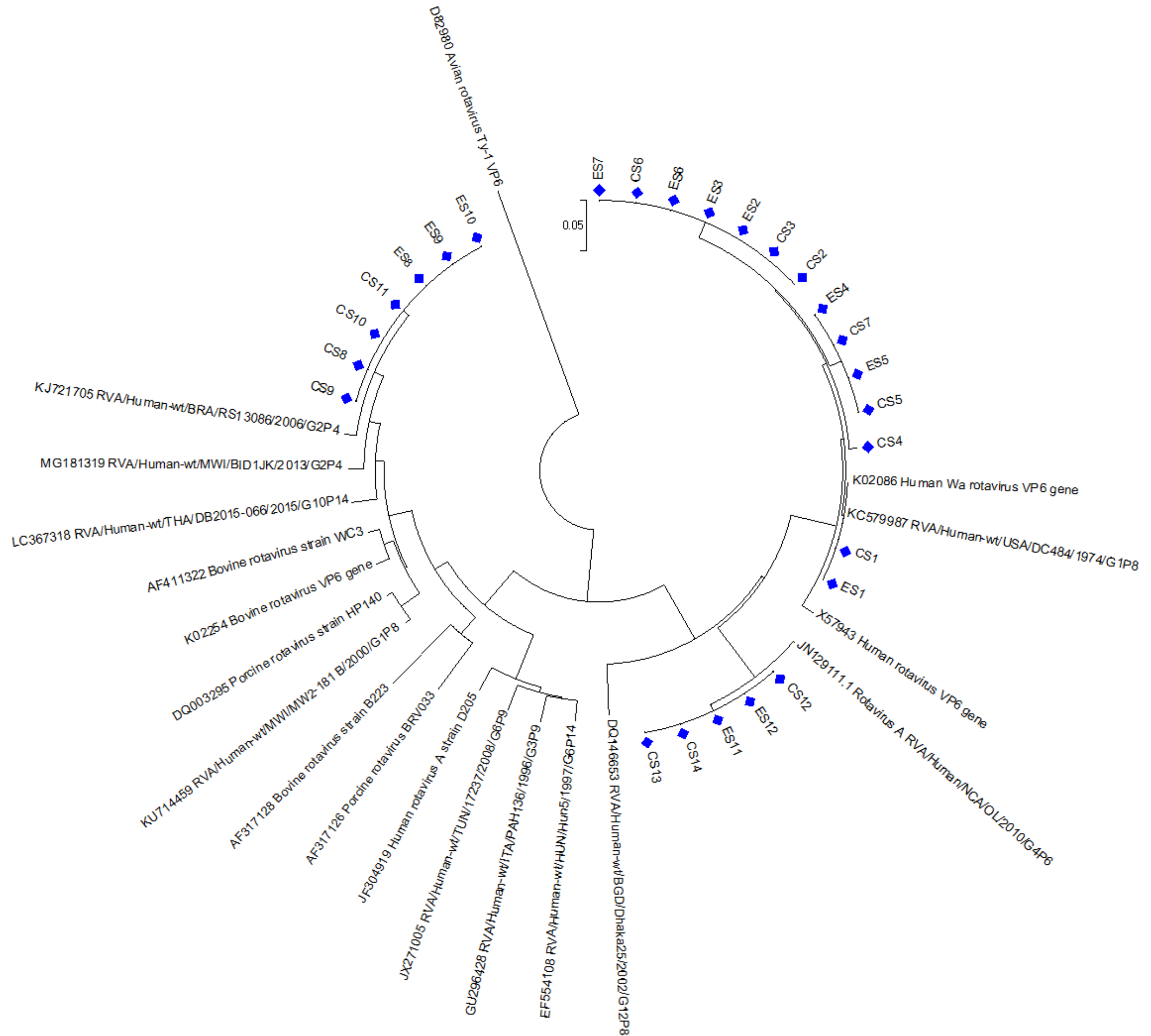

Fig. 3. Neighbour-joining tree constructed to represent the phylogenetic relationship between the nucleotide sequences of full length RV-A VP6 gene of environmental and clinical isolates (◆), human and animal rotavirus strains. All of the tested sequences clustered with human rotavirus G1P8, G2P4 or G4P6. Evolutionary distances were determined by the Kimura 2-parameters.

DISCUSSION

In this study, RoV VP6 was detected in 22.61% (232/1026) of the clinical diarrheal specimens collected during two years and using single round RT-PCR followed by sequencing for positivity conformation. The same clinical specimens were investigated previously in another study of our group (Food-Borne viruses group, NRC) (El-Senousy *et al*., 2020) using two rounds (Nested RT-PCR) with higher percentage of positivity 24.37% (250/1026) of rotavirus VP6. This may return to the higher sensitivity of nested RT-PCR than the single round RT-PCR. Although, eighteen clinical specimens showed false negative results using the single round RT-PCR, the general distribution of RoV through the year was not affected. The peak of RoV distribution in our present study was in autumn and winter which is consistence with the results of several previous studies (Levy *et al*., 2008; Chang *et al*., 2015).

In the present study, RoV had high detection rate in raw sewage samples. RoV was detected in 88.57% (31/35) of the two studied WWTPs with tendency to slightly higher frequency in WWTP-2 90.48% (19/21) than WWTP-1 85% (12/14). This could be due to the higher amount of raw sewage received by WWTP-2 (2.500 million m3/ day) than WWTP-1. Although, both WWTPs used activated sludge followed by final chlorine as treatment processes, poor removal of RoV was observed in both WWTPs. This may be because of the high number of genome copies of RoV in raw sewage, however the period of samples collection was in autumn and winter months which represent the frequency peak of the RoV. The high number of viral genome copies in raw sewage may be sometimes higher than the capability of the WTPs to remove viruses. The number of genome copies of RoV in Egyptian raw sewage in autumn and winter ranged from $7x10^3$ to $7x10^7$ genome copies/l (El-Senousy *et al*., 2013a; El-Senousy and Abou-Elela, 2017). The high prevalence of RoV in the treated sewage may be one of the reasons of the high frequency of RoV in the raw Nile water samples which collected in the same period of time.

In several previous studies in Egypt, RoV group A was the highest frequent among the RNA enteric viruses and second only to adenovirus in Egyptian raw sewage and raw Nile water samples. Also, it was the most resistant among the RNA enteric viruses and second to adenovirus to water and wastewater treatment processes. The high resistance of RoV to chlorine disinfection was previously reported (Bosch *et al*., 1993; Abad *et al*., 1994). The appearance of RoV genome six times out of 14 in drinking water samples may indicate the resistance of RoV to water treatment processes and chlorine disinfection. Another reason might be the high number of RoV genome copies in the raw Nile water which may be higher than the capability of WTP to remove the viruses. Presence of RoV genome copies in drinking water samples does not necessarily indicate viral infectivity in the samples, however PCR cannot discriminate between infectious and non-infectious pathogens. The testing capability is inconsistent with infectivity of the virus (Li *et al*., 2002; El-Senousy *et al*., 2007; Parshionikar *et al*., 2010; Hamza and Bibby 2019). Although RotaRix vaccine was available in Egypt more than ten years ago and after that Rotateq vaccine became available, RoV prevalence was still high in both clinical specimens and environmental samples in comparison to its prevalence before the commercial vaccines availability in Egypt (Villena *et al*., 2003; El- Senousy *et al*., 2004; El-Senousy and ELmahdy, 2009; El-Esnawy et al., 2010; El-Senousy *et al*., 2013a; El-

Senousy *et al*., 2013b; El- Senousy *et al*., 2014a; El-Senousy and Abou-Elela, 2017; El-Senousy *et al*., 2020). One explanation of this might be the fact that RoV vaccines are not in the free obligatory immunization program for children in Egypt and the relatively high price of the vaccines in the private sector. So, high percentage of children could not obtain the vaccines and consequently, they could not be vaccinated. In addition to the high cost of the two live attenuated vaccines, these vaccines have been associated with a low risk of intussusception; which is believed to be triggered by the replication of the oral live vaccine (Carlin *et al*., 2013; Weintraub *et al*., 2014; Yih *et al*., 2014; Yen *et al*., 2016). Also, these vaccines might be associated with other issues related to live vaccines including the risk of introduction of vaccine strains into the environment, genetically assortment between the vaccines strains and wild type strains, and the reversion of vaccine strains towards virulence (Lappalainen *et al*., 2015). This has stimulated interest in an alternative non-living parental approach to vaccination. Although genotypes G1P8 and G1P4 were the highest prevalent genotypes in both Egyptian clinical specimens and environmental samples in the last twenty years, high diversity of other genotypes were observed in several studies (Villena *et al*., 2003; El-Esnawy *et al*., 2010; El-Senousy *et al*., 2014a; El-Senousy and Abou-Elela, 2017; El- Senousy *et al*., 2020). This may suggest the VP6 recombinant subunit vaccine as a candidate RoV vaccine in Egypt and other countries with high diversity of RoV genotypes. Therefore, the main objective of our present study was to estimate silent and non-silent mutations in the whole VP6 amplified gene of Egyptian clinical specimens and environmental samples.

Four groups of sequences of clinical specimens and environmental samples which contained G1P8 genotype were observed. One of them which contained 1 clinical specimen and 1 raw sewage sample had similar sequences and showed 99.85% nucleotide identity and 99.75% amino acid identity with human RoV Wa reference strain. The second group contained 1 clinical specimen with 19 nucleotide substitutions (18 of them were silent and only one mutation was non-silent and caused one amino acid change). It showed 98.56% nucleotide identity and 99.75% amino acid identity with human RoV Wa reference strain. The third group which contained 3 clinical specimens and 4 environmental samples (1 raw sewage sample, the treated effluent of the same WWTP, 1 raw Nile water sample and the treated drinking water of the same WTP) had similar sequences with 32 nucleotide substitutions (25 silent and only 7 non-silent mutations) resulted in 7 amino acid changes giving 97.58% nucleotide identity and 98.24% amino acid identity with human RoV Wa reference strain. The fourth group, which contained 4 samples (2 clinical specimens, 1 raw sewage sample, and the treated effluent of the same WWTP) had similar sequences with 25 nucleotide substitutions (18 silent and only 7 non-silent mutations) resulted in 7 amino acid changes giving 98.1% nucleotide identity and 98.24% amino acid identity with human RoV Wa reference strain. These low variations in amino acid sequences in the samples which contained G1P8 genotype may suggest the VP6 recombinant subunit vaccine as a candidate vaccine for RoV which contained G1P8 genotype. However, this genotype in parallel with G1P4 genotype represent the most frequent genotypes in Egyptian clinical specimens and environmental samples (Villena *et al.,* 2003; El-Esnawy *et al.,* 2010; El-Senousy *et al.,* 2014a; El-Senousy and Abou-Elela, 2017; El-Senousy *et al.,* 2020). In our previous study which concerned with the VP8 partial gene (El-Senousy *et al*., 2020) and using the same samples of our present study, low variations in amino acid sequences in the VP8 partial gene between the samples

contained G1P8 genotype in addition to the low number of samples contained non-silent mutations in comparison to the two RoV reference strains (human RoV Wa strain and human RoV A strain USA) were observed. The same situation was observed in the samples contained P4 genotype or samples contained P6 genotype either in the VP6 whole gene or VP8 partial gene. The high similarity in the nucleotides and amino acids between samples contained the same genotype of human RoV in the sequences of more than one gene (VP8 in our previous study and VP6 in our present study) may confirm the low genetic variations between human RoV strains contained the same genotype which circulating in Egypt.

In our present study, the amino acid identity between samples contained G1P8 genotype and samples contained G2P4 genotype ranged from 92.19 % to 93.7 %. On the other hand, the nucleotide identity between samples contained G1P8 genotype and samples contained G4P6 genotype ranged from 95.21% to 96.73%, while, the amino acid identity ranged from 95.21 % to 96.73%. Finally, the amino acid identity between samples contained G2P4 genotype and samples contained G4P6 genotype was 93.45%. More studies are needed to investigate the homotypic and heterotypic immunity of the whole VP6 gene recombinant subunit vaccine of the different genotypes of human RoV.

El-Senousy and co-workers (2013c) reported that a short fragment of VP6 gene (155bp) was used as a target for recombinant subunit vaccine of human RoV. The results of the present study will be a base for preparation of recombinant subunit vaccine based on the whole VP6 gene and examination of its sensitivity in vitro and in vivo by our group (Food-Borne viruses group, NRC).

CONCLUSION

RoV was highly abundant in Egyptian environment; therefore, RoV vaccine should be included in the obligatory immunization program for children in Egypt. Additionally, the high similarity in nucleotide sequences of RoV VP6 full gene and consequently the amino acid sequences between isolates which contained the same or different genotypes may suggest RoV whole VP6 gene as a candidate recombinant subunit vaccine in Egypt and other countries which have circulating RoV with the same VP6 sequence characteristics

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