

Molecular detection and phylogenetic analysis of Astrovirus and Noroviruses from sewage water in Diyala-Iraq

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Abstract

Background: Enteric viruses (EVs) are a diverse group of human pathogens primarily transmitted feco-orally, are a major cause of diarrheal disease in both developed and developing countries. Human Astroviruses (HAVs) and human noroviruses (HNVs) are shed in high numbers by infected individuals and persist for a long time in wastewater posing a serious threat to human health globally.

Objectives: Molecular detection and genotyping of HAVs and HNVs from wastewater samples collected from sewage water plants (SWPs) and draining canals (DCs) in Diyala province-Iraq.

Specimens and methods: This study was conducted in Diyala province-Iraq for the period from November 2019 to October 2021. A total of 100 water samples were collected from SWPs and DCs. Immunochromatographic assay (ICA) technique (Biozek, Netherlands) was used for direct detection of Rotaviruses, Noroviruses, and Astroviruses antigens. While the Hepatitis A virus antigen was detected by CerTest BioTEC (Spain). For molecular detection, the water samples were firstly ultra-centrifuged and the EV RNAs concentration were measured. Then viral nucleic acids (NA) were extracted and submitted for real time-Polymerase Chain Reaction (RT-PCR). Positive samples with high CT were further submitted for Conventional PCR (PCR-ThermoFisher (U.S.A.)). For sequencing, PCR products were sent for Sanger sequencing (Macrogen Corporation – Korea). The sequencing results of the targeted samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). A specific comprehensive tree was constructed. The observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn serve. Statistical analysis was done using the Statistical Packages for Social Science - Version 27), and P value was considered significant whenever it is equal or less than 0.05.

Results: Using the RT-PCR technique the detection rate of HAVs and HNVs in SWPs and DCs was 58% for each. The detection rates of HAVs and HNVs were insignificant associated with wastewater collection sites ($P=0.390$) as well as with source of wastewater ($P=0.774$). Furthermore, 82% of the wastewater samples had co-detection of more than one virus. The conventional PCR product of ORF2 gene of HAV electrophoretically yields a band of 343 bps, while that of the ORF1 and ORF2 gene of HNV GI yields a band of 329 bps and that of the GII yields a band of 446 bps. The phylogenetic tree analyses revealed that the local Iraqi NVGII strains 1,2,3,4 and 5 are belong to the genotype NVGII.4, while the local Iraqi NVGI strains 6 and 7 belong to NVGI. On the Astrovirus side, the Iraqi local isolates 1 and 2 are 100% identical to the reference strain MG970106.1 of the NCBI.

Conclusion: RT-PCR assay found a high detection rates of HAVs and HNVs in wastewater samples collected from sewage water plants and draining canals in Diyala province-Iraq. Conventional PCR, sequencing and phylogenetic analyses genotyped the local Iraqi isolates of these viruses. The study suggest that documenting sewage virome using molecular methods provides information for molecular epidemiology and may be useful in developing strategies to prevent further spread of viruses.

Keywords: Sewage water, Astrovirus, Norovirus Diyala province.

INTRODUCTION

Increasing human population leads to increased demand for agricultural products and water, wastewater re-use will be necessary, which will pose a risk for virus pollution of the environment and subsequent affects viral transmission (Sano et al., 2016). More than 100 species of enteric viruses have been identified in human feces and in sewage such as astroviruses, caliciviruses, enteroviruses, enteric adenoviruses, and rotaviruses (Fernandez-Cassi et al. 2018; Gerba et al., 2018; Hoque et

al., 2019). Probably because sewage water constitute a suitable environment for survive of viruses. These may include temperature, light, pH, salinity, organic matter, suspended solids or sediments, and air-water interface (Akiyoshi et al., 1997; Albert, 2007). Raw sewage contains a mass of viruses (Meleg et al., 2006), mostly belonging to enteric viruses, which can be shed by individuals and transmitted via the fecal-oral route. Studies have been successfully conducted to detect these viruses in sewage (Lodder et al., 2005; Fong et al., 2010; Fumian et al., 2011; Han et al., 2014). Several studies have documenting viral existence and diversity in sewage is a useful tool for monitoring viruses circulating in the community (Smith et al., 2016; Strubbia et al., 2019). Most of human enteric viruses have small RNA genomes making their detection difficult. Indeed, their relative abundance if compared to bacteria, phage, parasites present in the environment challenge their detection and identification (Cotten and Koopmans 2016; Nieuwenhuijse and Koopmans, 2017; Adriaenssens et al., 2018).

Human Astroviruses (HAstVs) are non-enveloped, positive-sense single-stranded RNA viruses, belong to the genus Mamastrovirus, family Astroviridae. Its genome consists of three open-reading frames (ORFs), ORF1a, ORF1b, and ORF2 (Marczinke et al., 1994). ORF1a and ORF1b encode the non-structural viral proteins, and ORF2 encodes the capsid protein precursor. According to the sequence analysis based on 348-bp partial ORF2 coding region (Ulloa et al., 2005), HAstVs can be divided into eight genotypes (HAstV-1 to HAstV-8). Of these, HAstV-1 has been recognized as the most frequent genotype throughout the world (De Benedictis et al., 2011), and the second common genotype differs in different regions (Mustafa et al., 2000; Silva et al., 2006). Although controversial, HAstVs are mostly prevalent in winter in temperate regions, but in the rainy season in tropics (Mustafa et al., 2000, Nguyen et al., 2008). Higher burden of HAstV diarrhea occur every other year. Regarding the incidence of different serotypes, HAstV-1 is by far the most prevalent type worldwide, accounting for over 50% of cases (Vu et al., 2017).

Noroviruses are a leading cause of acute gastroenteritis globally and are implicated in waterborne and foodborne outbreaks (Bartsch et al. 2016). Norovirus, belonging to the family Caliciviridae, can be classified into seven genogroups. Genogroup (G)I, GII and GIV have been detected in humans, and can be further subdivided into more than 40 genotypes (Vinje 2015). Epidemiological studies shown that norovirus outbreaks caused by the GII.4 genotype are common in health-care facilities, whereas outbreaks caused by the GI and non-GII.4 genotypes are associated with waterborne and foodborne transmission (Verhoef et al. 2010; Qin et al. 2016; Giammanco et al. 2018). Noroviruses are shed at high concentrations with feces into wastewater, which is usually discharged into aquatic environments. Waterborne outbreaks caused by norovirus have been reported and associated with drinking tap water (Giammanco et al. 2018), contaminated water well (Qin et al. 2016) and wastewater intrusion into the water distribution network (Moreira and Bondelind 2017). Using qRT-PCR. Noroviruses were detected in 44.4% of recycled water samples and 73.9% of sewage sludge samples. Norovirus GI.2 and GII.4 were identified in recycled water samples. Six different genotypes of GI (GI.1, GI.2, GI.5a, GI.5b, GI.6b and GI.7) and GII.17 were identified in sewage sludge samples (Kittigul et al., 2019). A variety of norovirus sequences was detected in sewage samples, with genogroup II being more prevalent. Interestingly, the pig-mucin capture enhanced not only the recovery of norovirus and rotavirus but also recovery of astrovirus, sapovirus and husavirus (Strubbia et al., 2019).

Specimens and methods:

This Cross sectional study was extended over the period from November 2019 to October 2021, in Diyala province-Iraq. A total of 100 water samples were collected from 11 SWPs and 5 DCs in a disposable tightly cupped plastic containers. It is worthy to mention that most of the tributaries of Diyala river are stemming up from Iranian boundaries North East Iraq. Ashnona (Khresan) sub-river is one branches of Diyala river and both cross Baquba city, the center of Diyala province. The most important draining canals which directly evacuated in Diyala river are Al- Mukdadia, Athnona, and Khan Beni Saad. The main drinking water plant is situated on Ashnona sub-river. The collected water samples were delivered to the laboratory by cool box. Direct detection of EV antigen within 3 hours of collection. ICA technique (Biozek, Netherlands) was used for direct detection of Rotaviruses, Noroviruses, and Astroviruses antigens. While the Hepatitis A virus antigen was detected by CerTest BioTEC (Spain). Water samples were kept at 4 0 C for further molecular analyses. For molecular detection, the water samples were firstly ultra-centrifuged at 30000 RPM for 30 minutes. Then the EV RNAs concentration were measured using Thermo Scientific Nanodrop 2000 (USA). The viral nucleic acids were extracted, Sacace Biotechnologies Real Time PCR Kit (Italy) technique was used for detection of Rotaviruses, Noroviruses, Astroviruses from water samples. The HAV was detected by Sacace Biotechnologies quantitative Real time PCR Kit (Italy). Conventional PCR (Thermo-Fisher (U.S.A.) Superscript TM III One — step PCR — System with Platinum Tm Taq Hig Fidelity DNA Polymerase) was used for viral NAs amplification.

Table (1): Specific primer for ORF2 of Astrovirus and ORF 1 and 2 of Astrovirus.

Primers	Sequence (3'-5')	Sense	Application size	Reference
Astrovirus				
Mon269	CAACTCAGGAAACAGGGTGT	+	448 bp	

Mon270	TCAGATGCATTGTCATTGGT	-		(Stacy, et al., 2009)
Norovirus				
GISKF	CTGCCCGAATTYGTAAATGA	+	329 bp	(Elisabeth, et al., 2011).
GISKR	CCAACCCARCCATTRTACA	-		
G2SKF	CNTGGGAGGGCGATCGCAA	+	343 bp	
G2SKR	CCRCNCGATRHCRTTRTACAT	-		

The PCR products were submitted for gel electrophoresis on agarose gel (3%) in TEB buffer using 3 volts/cm² stained by Redsafe NA staining solution. The patterns of bands migration was visualized by exposure to UV gel documentation.

For sequencing, PCR products were sent for Sanger sequencing using ABI3730XL, automated CDNA sequences, by Macrogen Corporation – Korea. The results were received by email then analyzed using Genious software. The sequencing results of the PCR products of the targeted samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome. The amino acid sequences of the targeted protein were retrieved online from the protein data bank (<http://www.ncbi.nlm.nih.gov>). The observed variants in the coding portions were translated into a reading frame corresponds to the referring amino acid residues using the Expasy online program (<http://web.expasy.org/translate/>). Multiple amino acid sequences alignment was conducted between the referring amino acid sequences and its observed mutated counterpart using the “align” script of the BioEdit server. A specific comprehensive tree was constructed according to the neighbor-joining protocol described by Hussein et al. (2020). The observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server (Zhang et al., 2000). Statistical analysis was done using the Statistical Packages for Social Science — Version 27), and P value was considered significant whenever it is equal or less than 0.05.

Results:

It was important to mention that the total detection rate of the four viruses included in this study; Rotavirus, Astrovirus, Norovirus and Hepatitis A virus in water samples collected from sewage water plants and draining canals using the RT-PCR technique and immunochromatographic assay (ICA) was 92.0% and 87.0% respectively. However, the detection rate of Astrovirus and Norovirus in sewage and draining canals water was 58% for each, table (1).

Table (1): Astrovirus and Noroviruses detection rate by ICA and RT-PCR.

Tested viruses	ICA results				RT-PCR results			
	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
Astrovirus	33	33	67	67	29	58	21	42
Norovirus	9	9	91	91	29	58	21	42

According to the collection site, the detection rates of both Astrovirus and Norovirus from water samples collected from sub-district areas was insignificantly higher than that of district areas (64.0% Vs 52.0%, P= 0.390).

Table (2): Detection rates of Astrovirus and Norovirus by RT-PCR according to collection areas

Tested virus	Sample collection sites				P value
	District		Sub-district		
	Positive (%)	Negative (%)	Positive (%)	Negative (%)	
Astrovirus	13 (52.0)	12 (48.0)	16 (64.0)	9 (36.0)	0.390 *
Norovirus	13 (52.0)	12 (48.0)	16 (64.0)	9 (36.0)	0.390*

*Insignificant difference between percentages using Pearson Chi-square test (χ^2 -test) at 0.05 level.

Table (3): Detection rates of Astrovirus and Norovirus by RT-PCR according to type of water.

Tested virus	Type of water				P value
	Sewage water plants		Draining canals		
	Positive (%)	Negative (%)	Positive (%)	Negative (%)	
Astrovirus	15 (60.0%)	10 (40.0%)	14 (56.0%)	11(44.0%)	0.774*
Norovirus	15 (60.0%)	10 (40.0%)	14 (56.0%)	11(44.0%)	0.774*

*Insignificant difference between percentages using Pearson Chi-square test (χ^2 -test) at 0.05 level.

The RT-PCR results also found that out of 50 tested water samples, 46 (82.0%) were containing more than one virus at the same time. The highest combination was found gathering Astrovirus and Norovirus (58.0%). furthermore, all the three viruses (Astrovirus, Norovirus and Hepatitis A virus) were detected in 30% of water samples. table (4).

Table (4): Co-detection rates of Norovirus and Astrovirus with other virus by RT-PCR.

Co-detected viruses	Positive	(%)	Negative	(%)
Norovirus +Rotavirus	13.0	26.0	37.0	74.0
Norovirus +Astrovirus	29.0	58.0	21.0	42.0
Norovirus +Hepatitis A virus	15.0	30.0	35.0	70.0
Norovirus +Rotavirus+ Astrovirus	12.0	24.0	38.0	76.0
Norovirus +Rotavirus+ Hepatitis A virus	5.0	10.0	45.0	90.0
Astrovirus+ Rotavirus+ Hepatitis A virus	6.0	12.0	44.0	88.0
Astrovirus+ Norovirus + Hepatitis A virus	15.0	30.0	35.0	70.0

The gel electrophoresis of the PCR product of the ORF 2 gene of Astrovirus yield a band of 343 bp, figure (1), while the gel electrophoresis of the PCR product of ORF1 and ORF 2 gene of Norovirus GI yeild a band of 329 bp as shown in figure (2). On the other hand, the gel electrophoresis of ORF1 and ORF 2 gene of Norovirus GII yield a band of 448 bp as shown in figure (3).

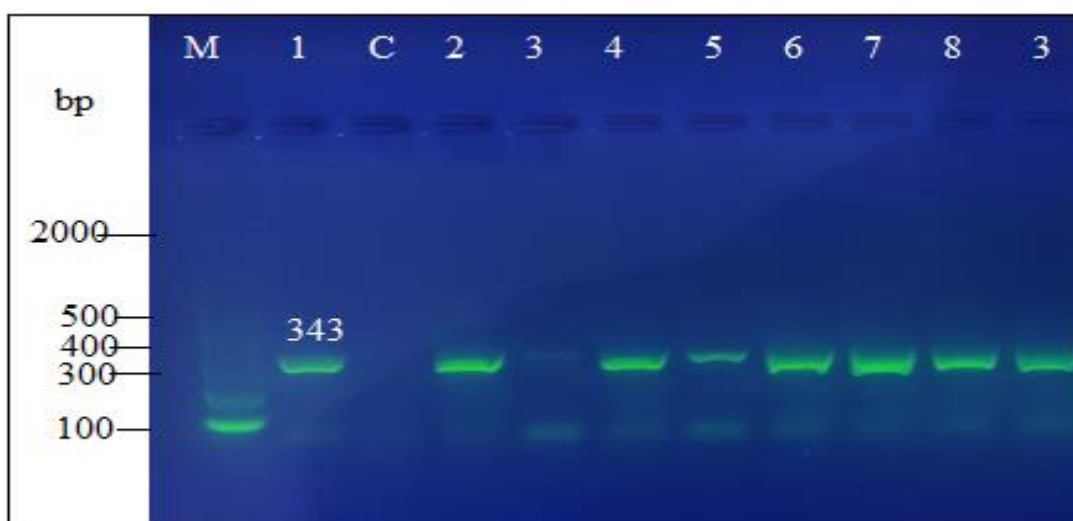


Figure (1): Gel electrophoresis of the ORF2 of Astrovirus migration pattern.

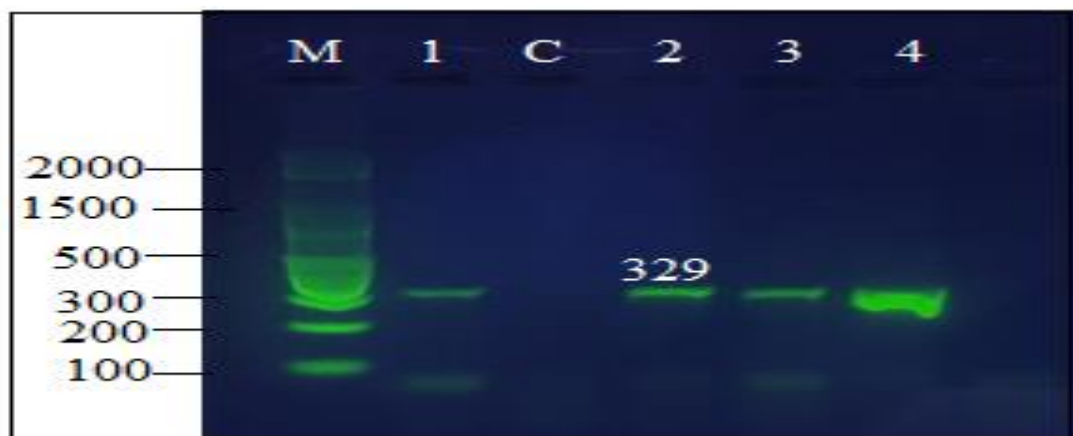


Figure (2): Gel electrophoresis of the ORF1 and ORF 2 gene of Norovirus GI migration pattern.

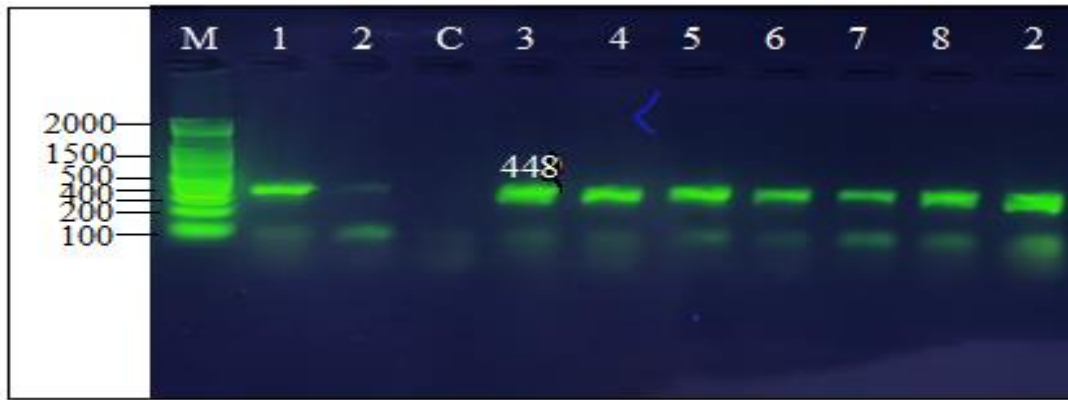


Figure (3): Gel electrophoresis of the ORF1 and ORF 2 gene of Norovirus GII migration pattern.

A specific comprehensive tree was constructed according to the Maximum Likelihood method and Tamura-Nei (Tamura and Nei, 1993) through the investigation of the basepairs sequences of local strains of Norovirus and Astrovirus. The observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server. This analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 356 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

The results found that the local Iraqi isolates of norovirus 1,2,3,4 and 5 belong to the genotype NVGII.4 which is common in Asian countries e.g. Chania, Japan, Russia and also reported in USA and Brazil. While the Iraqi Norovirus isolates 6 and 7 belong to the genotype NVGI, which is less common the NVGII.4 genotype with 100% identical to Russian and Taiwanese isolates.

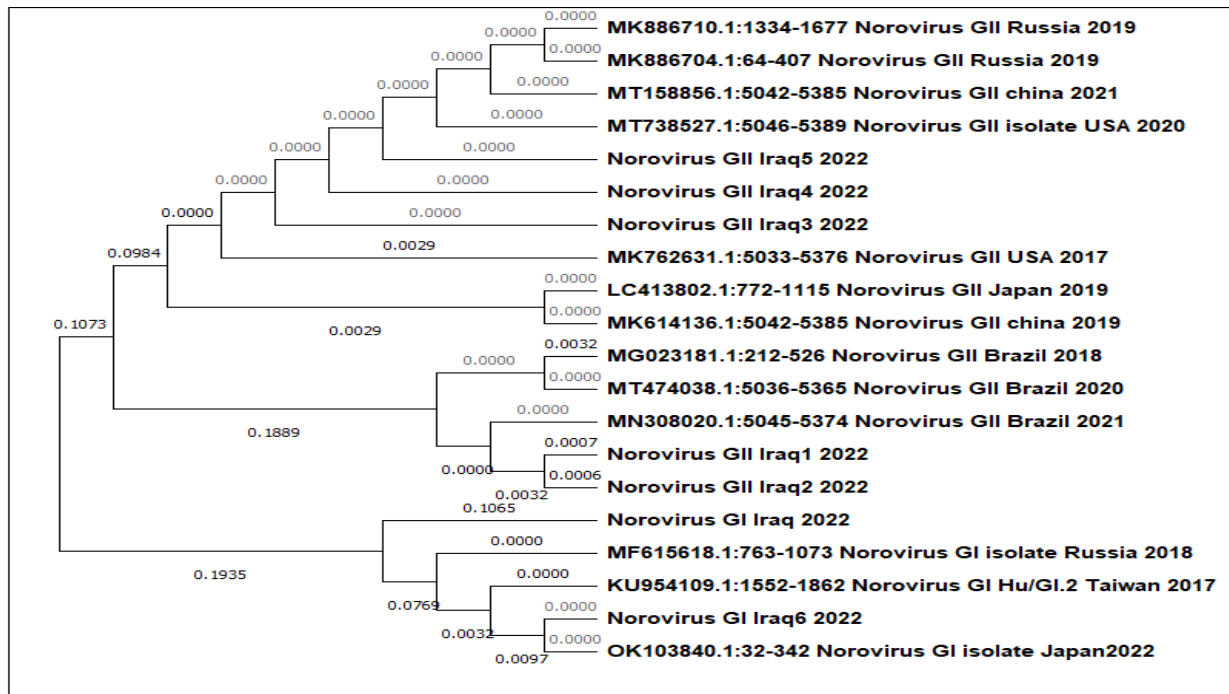


Figure (4): The comprehensive cladogram phylogenetic tree of Norovirus detected from sewage water.

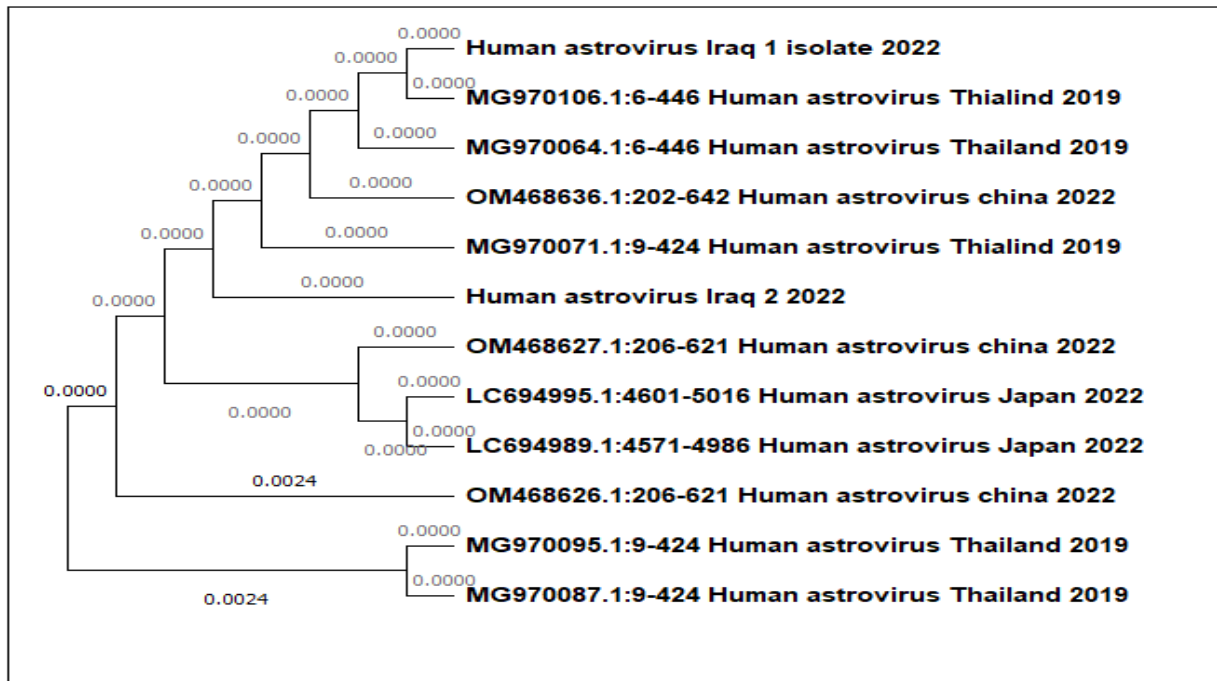


Figure (5): The comprehensive cladogram phylogenetic tree of Astrovirus detected from sewage water.

Discussion:

Human enteroviruses (EVs), comprising more than 100 serotypes, which are associated with a variety of clinical presentations such as acute flaccid paralysis (AFP), meningitis, encephalitis, cardiac disease, pleurodynia, acute hemorrhagic conjunctivitis, hand-foot-and-mouth disease and others (Pallansch et al., 2013). Environmental contamination occurs through discharge of waste materials from infected or carrier persons. Thus environmental surveillance mostly by examining sewage specimens contaminated by human feces (Bosch, et al., 2006). Surveillance through molecular detection of enteroviruses by quantitative real-time PCR method on sewage has been demonstrated to be an effective approach in investigating local circulation of enteroviruses especially in countries with limited disease-based EV surveillance system (Strubbia et al., 2019). Since, no previous such studies were conducted in Iraq, so the current study was suggested.

The total detection rate of Evs in SWPs and DCs was 92% using the RT- PCR and ultracentrifugation to increase the viral NAs concentration without cell culture. Furthermore, the detection rate of Astrovirus and Norovirus 58%, for each. The close similarity of the detection rates obtained by ICA and PCR techniques may endorse each other as the ICA technique has high sensitivity and the PCR technique characterize by high sensitivity and specify (Zhou et al., 2012; Garibyan and Avashia, 2013). Therefore, the high detection rate of both HAVs and HNVs reflects that these viruses circulated at a relatively high frequency in the local population, and demonstrates that environmental surveillance is an effective method in investigating circulating EVs (Zhou et al., 2014; Smith, et al., 2016). Actually these high molecular detection rates are supported by the high infection rate of these viruses in Iraqi community as detected in the clinical specimens by immunological assays (Al-Marsome et al., 2016; Ali, et al., 2016; Al-Tabtabai et al., 2020).

Norovirus is the leading cause of sporadic cases and outbreaks of acute gastroenteritis in children and adults (Patel et al., 2008; Bartsch et al. 2016; Bartnicki et al., 2017). The genera Norovirus belongs to the Caliciviridae family are further classified into ten distinct genogroups (GI–GX) that are subdivided into different genotypes (Chhabra et al., 2019). Genogroup (G)I, GII and GIV have been detected in humans, and can be further subdivided into more than 40 genotypes (Vinje 2015). Genogroups GI, GII, GIV, GVIII, and GXIX have been identified in infecting humans. Most of the human isolates belong to genogroups GI and GII that are further subdivided in 36 genotypes (GI.1-9, GII.1-28, and GII.15 has been withdrawn) (Chhabra et al., 2019).

There is a general consensus that Noroviruses are widely distributed in sewage water (Lodder and de Roda Husman , 2005; Eftim et al., 2017; Tiwari and Dhole, 2018). In Spain, Santiso-Bellon et al. (2020) reported that Noroviruses are detected in sewage water (69.6% for norovirus GI, 76.0% norovirus GII). 6.25% of the samples contained GI.2 genotype, and another 6.25% were positive for GI.4 genotype. The most common genotype of GII noroviruses was GII.2 (40%), followed by GII.6 (8.6%) and GII.17 (5.7%) while the remaining GII strains could not be typed (45.7%). Suggesting that the surveillance of

noroviruses in sewage is useful for the study of their transmission in the population and their molecular epidemiology. Clinically, epidemiological studies shown that norovirus outbreaks caused by the GII.4 genotype are common in health-care facilities, whereas outbreaks caused by the GI and non-GII.4 genotypes are associated with waterborne and foodborne transmission (Verhoef et al. 2010; Qin et al. 2016; Giammanco et al. 2018). Therefore, there is a close relation among the serotypes of enterovirus shed in stools and isolated from the environment with those detected in sewage samples revealing the relevant epidemiological data on the distribution and persistence of human pathogenic viruses in sewage-polluted waters and addresses the potential health risks associated with transmission of these viruses through water-related environmental routes (Rodríguez-Díaz et al., 2009).

In Brazil, Using RT-PCR, norovirus GI and GII was detected in 38.5% and 96.1% of raw sewage samples, 40.4% and 96.1% of primary effluent samples and 1.9% and 5.8% of final effluent samples, respectively. GII.4 noroviruses were the most prevalent in wastewater samples (68.5%), and a similar trend was observed in acute gastroenteritis cases (71%). The emergent GII.17 was the second most prevalent genotype (14.3%) identified in the raw sewage samples, however, it was not detected in clinical cases (Fumian et al., 2019). In another study, Noroviruses were detected in 444% of recycled water samples and 73.9% of sewage sludge samples. Norovirus GI.2 and GII.4 were identified in recycled water samples. Six different genotypes of GI (GI.1, GI.2, GI.5a, GI.5b, GI.6b and GI.7) and GII.17 were identified in sewage sludge samples. Four recombinant norovirus strains were detected in sewage sludge samples, namely GII.P16- GII.2, GII.P16-GII.4, GII.P16-GII.13 and GII.P21-GII.13. Providing evidence that noroviruses may be spread to the community and environment via the use of recycled water for plant areas, and sewage sludge for land application (Kittigul et al., 2019).

Molecular and phylogenetic analyses of PCR product of the ORF 2 gene of local isolates of Astrovirus yield 2 isolates were belong to the local Iraqi strains and the remaining 10 were belong to stains in the NCBI with 100% identical with the reference strain MG970106.1 which is common in Thailand, Chain and Japan.

Since their first discovery in 1975, human astroviruses (HAsVs) have been well-known as etiological agents of viral gastroenteritis with a worldwide distribution (Mendez and Arias, 2007). They are small, non-enveloped, single-stranded positive RNA viruses and they make up the Astroviridae family. To date, the family has been divided into two genera: Mamastrovirus and Avastrovirus, including viruses infecting mammals and birds, respectively. Their genome codes for three ORFs, with ORF1a and ORF1b encoding the nonstructural protease and polymerase proteins, respectively, and ORF2 encoding the capsid proteins (Vu et al., 2017).

Since 2008, two novel groups of highly divergent astroviruses, named MLB (Melbourne) and VA/HMO (Virginia/Human-Mink-Ovine-like), have been identified in human stool of individuals with diarrhea using next-generation sequencing (NGS) (Finkbeiner et al., 2008).

Classical HAsVs are classified into 8 serotypes (HAsV-1 to HAsV-8) with 64%–84% capsid amino acid similarities. Within each serotype, different genetic lineages or subtypes were identified, based on a lower than 93%–95% nucleotide homology of partial ORF2. Lineage classification was reviewed (Martella et al., 2014), with 6 lineages within HAsV-1 (1a to 1f), 4 within HAsV-2 (2a to 2d), 2 within HAsV-3 (3a and 3b), 3 within HAsV-4 (4a to 4c), 3 within HAsV-5 (5a to 5c), and 2 within HAsV-6 (6a and 6b). The subsequently-identified HAsV-3c was added to the classification (Medici et al., 2015). Compared to classic HAsVs, novel HAsVs are even more diverse. MLB-HAsVs (Mamastrovirus 6) is classified in 3 types or clades (MLB1, MLB2, and MLB3), while VA HAsVs are divided in Mamastrovirus 8 species, containing VA2 (also named HMO-B) and VA4, and Mamastrovirus 9 species containing VA1 (also named HMO-C) and VA3 (HMO-A) (Jiang et al., 2013).

The detection of HAsVs in sewage water and DCs water samples in the current study is similar to a lot of studies worldwide (Pinto et al., 2001; He et al., 2011; Prevost et al., 2015; Lizasoain et al., 2018; Shaheen et al., 2018). Furthermore, Cann et al.(2004), reported that the high prevalence of astroviruses in sewage treatment plant affluents and effluents indicates that these plants are not efficiently eliminating the virus.

Probably one reason behind the thigh detection rates of HAsVs in sewage water is the high genetic diversity of Astrovirus potentiating infection of a large spectrum of mammals and birds (De Benedictis et al., 2011). The high genetic variability of HAsVs, together with the occurrence of recombination events during concurrent infections with multiple strains, makes them serious candidates for emerging zoonotic infections. Cross-species transmissions are especially frequent in avian viruses (Battisti et al., 2012). Supporting this notion, In China, genetic cloning and sequencing of positive PCR products of HAsVs were phylogenetically analyzed revealed 4 genotypes (HAsV-1, -2, -4 and -5), with HAsV-1 and -5 as the most common genotypes respectively. The high detection rate reflects that HAsVs circulated at a relatively high frequency in the local population, and demonstrates that environmental surveillance is an effective method in investigating circulating HAsVs (Zhou et al., 2014). A convincing evidence that HAsVs circulate at a relatively high frequency in the Hungarian population based on high detection rates of these viruses in sewage water was reported by (Meleg et al., 2006). In Egypt, the detected higher titers of genogroup B may indicate the emergence of genogroup B which exhibited a higher resistance to removal

treatments from sewage water (El-Senousy et al., 2007).

The study concluded that a detection rate of both HNVs and HAVs from wastewater samples collected from sewage water treatment plants and draining canals in Diyala-Iraq. At the present time with almost totally non-functional sewage water treatment plants, similar periodic surveillance studies are required to monitor of public health risks of these enteroviruses.

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