JJBS
Jordan Journal of Biological Sciences

Volume 13, Number 3, September 2020
ISSN 1995-6673
Pages 327 - 335

Molecular Surveillance of Enteroviruses in Al-Zarqa River, Jordan

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Received June 18, 2019; Revised September 7, 2019; Accepted September 16, 2019

Abstract

This study is concerned with Al-Zarqa River, one of the major water resources in Jordan. It investigated its surface water quality with respect to the occurrence and serotypes of enteroviruses in different water samples collected from the river over a period of 11 months. Viruses were concentrated from river water, raw sewage and effluent samples with a calculated % recovery yield ranged between 2% and 8%. The concentrations of enteroviruses ranged between 3.3 and $6.3X10^3$ pdu/ml, with a mean of $2.5X10^3$ pdu/ml in raw sewage. Thirty-three samples were examined for the presence of enteroviruses by means of RT-PCR and southern blotting hybridization. Enteroviruses were detected in 14 (42%) of the samples. Sequence analysis of RT-PCR products followed by phylogenetic analysis revealed the frequent detection of coxsackievirus B4 and poliovirus serotype type 1, while the remainder comprised coxsackievirus B3, echovirus 9 and echovirus 11. Enteroviruses sequences isolated from samples collected from different sites along the river were similar, but differences were observed in samples collected from the same sites at different times. Circulation of enterovirus in sewage and water, serotyping and phylogenetic analysis enabled us to trace back the source of enterovirus contamination.

Keywords: Enterovirus; phylogeny; serotype; Zarqa River; wastewater

1. Introduction

It is estimated that, more than 100 different types of potentially pathogenic microorganisms that may cause an immense range of diseases and clinical symptoms can be present in polluted water (Iwai *et al.*, 2006). Transmission of pathogens via the environment can clearly result in infections and diseases to both humans and animals, resulting in the circulation of pathogens in the environment (Westrell, 2004). Heavy rains can cause run-offs from agriculture land and will reintroduce pathogens from sludge and manure into rivers, which can contribute substantial loads of pathogens (Hansen and Ongerth, 1991; Kistemann *et al.*, 2002; Ferguson *et al.*, 2003).

Al-Zarqa River is the only inland river in Jordan, and is the second main tributary to River Jordan after Yarmouk River. The pathogens of main concern in the Al-Zarqa River basin include bacteria, protozoa and viruses with the latter being of main concern as they generally pose a greater challenge than bacteria and protozoa in food and wastewater treatment (Sibanda and Okoh, 2012). The risk of viral infections can be 10 to 1,000 times higher than that for bacteria at a similar level of exposure (Rose and Gerba, 1991). In addition, viruses can survive in water longer than bacteria, and can tolerate greater variations in temperature and pH [6, 8] (Maunula *et al.*, 2005; Sibanda and Okoh, 2012). Therefore, the presence of waterborne viruses as enteric viruses in sewage and surface waters is considered a good indicator of the infectious status of a population, public health in general, (Sibanda and Okoh, 2012), and water safety and quality (Pina *et al.*, 1998). Furthermore, viruses are more resistant to commonly employed water and wastewater disinfection solutions. Thus, viruses can easily be harbored in "microbiologically immaculate" water (Payment *et al.*, 1994; Leeds, 2002).

In Jordan, many cases of enteric viral diseases, such as gastroenteritis, hepatitis A and aseptic meningitis have been reported (Toukan *et al.*, 1988; Meqdam *et al.*, 1997a, 1997b; Battikhi, 2002; Meqdam *et al.*, 2002; Battikhi and Battikhi, 2004; Nimri *et al.*, 2004; Khuri-Bulos and Al Khatib, 2006). These studies indicated that there is a direct or indirect possibility of viral occurrence in and/or viral infection through water environment. Three independent studies were carried out to investigate the enteroviruses prevalence in Jordan (Reichler *et al.*, 1997a, 1997b; Meqdam *et al.*, 2002). However, all these studies were based on detecting enteroviruses from clinical samples collected from children and adults. Another study performed by Malkawi and Shaban (2007) aimed to detect enteroviruses in different domestic water resources such as home water tanks and wells.

The very limited data on viral occurrence in water in Jordan makes it difficult to determine the health risks associated with these viruses. Moreover, to prevent diseases induced by waterborne viruses, a survey of viral occurrence in the water environment is badly needed. Therefore, in this study, Al-Zarqa River, one of the major

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water resources in Jordan, was chosen in order to investigate its surface water quality with respect to the presence of waterborne human pathogenic viruses, especially the enteroviruses. The study is considered as the first to investigate the types of enteroviruses in water environmental samples collected from Al-Zarqa River, with a special focus on the serotypes and phylogeny of enteroviruses.

2. Materials and Methods

2.1. Site Location and Background.

Aquifers and basins represent Jordan's primary sources of water. Amman-Zarqa Basin is the second largest basin after The Yarmouk Basin with an area of about 3,900 km^2. The basin is located in the most densely populated area in Jordan, accounting for 65% of Jordan's population and 90% of the industries in the country (Arab Environment Monitor (AEM) 2006). In addition, the basin provides irrigation for 8,400 hectares of land around. With the high population growth and high demand on water; the basin is subject to increased exploitation of its natural base-flow to an irreversible state (AEM 2006). Moreover, the annual effluent of treated domestic and wastewater treatment plants (total of 60 million cubic meters) has been contributing to nearly all of its summer flow (Optimization for Sustainable Water Resources Management (OPTIMA) 2006).

As-Samra Wastewater Treatment Plant (ASWWTP) (Fig. 1) is heavily overloaded with domestic wastewater effluent from the Amman-Zarqa basin containing high organic loads, plus industrial wastewater containing chemical and organic pollutants. Furthermore, ASWWTP receives domestic wastewater from about 2 million inhabitants in Jordan. Due to this high load, ASWWTP performance has been deteriorating over the years, resulting in lowering the effluent quality and deteriorating the groundwater quality in Zarqa basin to a level that is unsuitable for irrigation (AEM 2006), thus undermining the quality of life of residential communities in the Amman-Zarqa basin (Westrell 2004; De Luca *et al*., 2013).

2.2. Collection of Raw, Treated Sewage, Surface Water and Pond Water Samples.

A total of 33 samples of raw sewage, treated sewage, surface and pond water were collected over a period of one year (Table 1). All the 33 water and effluent samples were collected from places along the Zarqa River at different distances from the ASWWTP effluent to study the types and quantities of enteroviruses. Figure 1 presents a summary of the samples and the map shows physically the places along the river where the samples were collected. Samples were kept cool and processed as soon as possible or stored at -70 °C until processed.

Table 1. The location, type, number and the volume of the samples collected along Al-Zarqa River.

Site	Description of the site	Type of sample	No. of samples
A	ASWWTP inlet	Composite Raw sewage	3
B	ASWWTP outlet	Composite Treated water	3
C	2 km downstream the ASWWTP, near Al-Hashemia city	Surface river water	7
D	11 km downstream the ASWWTP, Al-Sukhna town	Surface river water	7
E	Al-Sukhna town bridge	Untreated water	3
F	40 km downstream the ASWWTP, Jerash bridge	Pond water	3
G	40 km downstream the ASWWTP, Jerash bridge	Surface river water	7
		Total	33

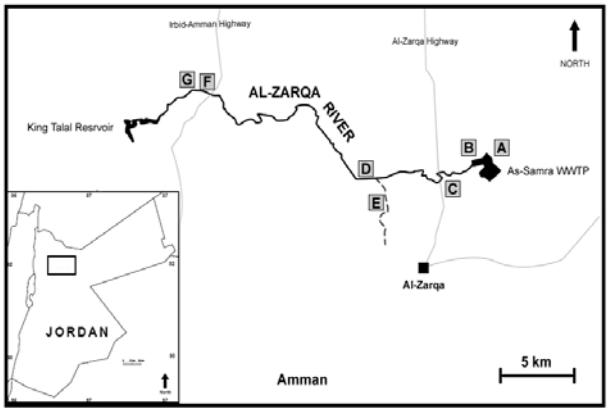

Figure 1. Locations of sampling sites along Al-Zarqa River. A: ASWWTP inlet; B: ASWWTP outlet; C: 2 km downstream the ASWWTP, Al-Hashemia city; D: 10 km downstream the ASWWTP, Al-Sukhna town; E: Al-Sukhna town bridge; F: 40 km downstream the ASWWTP, Jerash bridge, Jordan Modern Private Nursery; G: 40 km downstream the ASWWTP, Jerash bridge, Jordan Modern Private Nursery.

2.3. Concentration of Samples.

Surface water samples were concentrated by the adsorption-elution method (Haramoto et al. 2005) with modifications. Seven to eight liters of surface water samples were first allowed to reach the room temperature; the pH was then adjusted to 3.5 with 0.1 N NaOH (Frutarom, UK). The adjusted samples were pumped through a glass filter (Millipore, Ireland) and 0.45 µm mixed cellulose ester (Millipore, Ireland). Filter-adsorbed viruses were eluted with sterile 200 ml of 0.1% skimmed milk (Oxoid, UK) in 0.05 M Glycine buffer (pH 9.5) (Biorad, Italy) and kept in sterile bottles. Further concentration was done by organic flocculation after lowering the pH of the eluent to 4.5, followed by centrifugation at 4500 rpm for 30 min at 12°C. The resulting pellet was resuspended in a 10 ml sterile Phosphate buffered saline (PBS) (pH 7.2) (Lonza, Belgium) and stored at -70°C until subjected to RNA extraction.

Composite raw and treated sewage samples were concentrated by a two-phase separation method described by van den Berg *et al.* (2005). The average concentrate

volume was 2 ml and it was stored at -70°C until subjected to RNA extraction.

2.4. Calculation of Virus Recovery.

The recovery percentage of the applied concentration methods used in this study was calculated by spiking the collected water and sewage samples with the bacteriophage PRD1, provided kindly by The National Institute of Public Health and the Environment (RIVM), The Netherland. The bacteriophage PRD1 was used to evaluate the efficiency of virus recovery. First, surface water, treated water and raw sewage were spiked with 10^6 pfu of PRD1, and then the concentration procedures were performed as described earlier. Both, the un-concentrated samples and resulting concentrates were tested for the presence of PRD1 using the double-agar-layer method as described in ISO 10705-1 (ISO, 1995). The percentage of recovery was calculated using the following formula:

$$\%\ Recovery = \frac{No.of\ PFU \times volume\ of\ concentrate}{No.of\ PFU \times volume\ of\ spiked\ sample}$$

2.5. RNA extraction, RT-PCR amplification and gel electrophoresis.

Viral RNA was extracted from retenates using the silica beads in the presence of guanidinium isothiocyanate (Fluka, Switzerland) following the method developed by Boom et al. (1990). Primers and probes used were purchased from Alpha DNA (Canada), Midland (Canada) and Invitrogen (Germany). These primers were E1: 5´-CCTCCGGCCCCTGAATG-3´ and E2: 5´-ACCGGATGGCCAATCCAA-3´ (Sheih *et al.*, 2003). A 18 µl anti-sense mix composed of 15 µl of extracted RNA plus 50 pmole of the reverse primer E2 and 16 U from the RNAse inhibitor RNsin (Promega, USA) was heated at 95°C for 5 min, then chilled on ice for at least 5 min. To make the cDNA, a 12 µl reverse transcription mix containing 1X PCR buffer (10 mM Tris–HCl [pH 8.3]), 3 mM $MgCl_2$, 1 mM dNTP's and 5 U AMV-RT (All from Promega, USA) was combined with the heated anti-sense mix and incubated at 42°C for 1 h followed by 5 min incubation at 99°C then on ice for at least 5 min. PCR was performed according to van den Berg *et al*., (2005) by adding 15 µl of cDNA, the forward primer E1, 1X PCR buffer (10 mM Tris–HCl [pH 8.3]), 1.5 mM $MgCl_2$, 0.2 mM dNTP's and 5 U of Taq Polymerase (All from Promega, USA). PCR cycling was as follows: initial denaturation for 3 min at 94°C, 45 cycle of 1 min at 94°C, 1 min at 37°C and 1 min at 72°C followed by a final extension step for 10 min at 72°C (Applied Biosystems, USA). PCR products were separated onto 2% agarose (Biobasic, Canada) in the presence of 1 µg/ml ethiduim bromide (Promega, USA).

2.6. Extraction of PCR products from gels.

After electrophoresis and separation of all PCR products on 2% agarose (Biobasic, Canada), gels were visualized under UV illumination. Purification of PCR products from the gel material was done either using the Qiaquick gel extraction kit (Qiagen, Germany) or the DNA isolation kit (AppliChem, Germany) following the manufacturer's instructions.

2.7. Southern-Blotting Hybridization.

Agarose gels containing the RT-PCR products were first soaked for 1 h in 2 times the gel volume with denaturing solution, neutralized with the same volume of 1X neutralizing solution, and finally soaked in 20X Saline, Sodium Phosphate, EDTA (SSPE) buffer (Lonza, Belgium) for 3 h as described by (Lodder and Husman, 2005). Transfer was made in 20X SSPE to a positively charged nylon membrane (Millipore, Ireland) by using a home-made capillary transfer apparatus. Next day, the membrane was washed with 5X SSC (Lonza, Belgium) two times 5 min each. After that, the transferred DNA was cross-linked by heating the membrane in the microwave (Sharp, Japan) for 8 min. Subsequently, the membrane was pre-hybridized at 55°C with 20 ml of 2XSSPE-0.1%SDS (Promega, USA) for at least 1 h and then hybridized overnight at 55°C with a fresh 20 ml of 2XSSPE-0.1%SDS containing 40 pmole of the biotinlyted EV probe (5´-ACTACTTTGGGTGTCCGTGTTTC-3´) (Sheih *et al.*,2003), which is specific for the 5' untranslated region. Detection was made using biotin chromogenic detection kit (Fermentas, EU) following the manufacturer's instructions.

2.8. Calculation of the Enteroviruses RNA Concentration.

The number of virus particles present in all samples was estimated semi-quantitatively by RT-PCR and southern blotting on ten-fold serially diluted RNA (end point dilution) as previously described by (Rutjes *et al*., 2005). The estimation of the virus RNA concentration (PCR detectable unit per liter [pdu/l]) in the water was based on the most diluted sample, which gave a positive signal after hybridization of the RT-PCR products. Five ml from each sample concentrate (except for the raw sewage samples, where only 1 ml) was used to extract RNA; the final extracted volume was 15 µl. The extracted RNA 10-fold diluted up to 10^{-4}. All the undiluted and the diluted RNA were subjected to RT-PCR then Southern blotting as described earlier.

2.9. Cloning.

Southern hybridization positive products were cut from the gel with a clean scalpel and purified using the NucleoSpin Extract II kit (Macherey-Nagel, USA). The purified products were then inserted into the pJET1.2/blunt cloning vector provided in the CloneJET PCR cloning kit (Fermentas, EU) according to the manufacturer's instructions. The resulting vectors were transformed into *Escherichia coli* GW2163 (Fermentas, EU) using the TransformAid bacterial transformation kit (Fermentas, EU). Transformed bacteria were selected on 37°C pre-warmed LB agar plates containing 50 µg/ml ampicillin and incubated overnight at 37°C. Next day, 10 colonies from each plate were inoculated into LB broth containing 50 µg/ml ampicillin and incubated for 12-16 hours at 37°C with shaking at 120 rpm. After that, plasmids were extracted from the overnight culture using the GeneJET Plasmid Miniprep Kit (Fermentas, EU). PCR using the primer pair pJET1.2 forward sequencing primer (5'-CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2 reverse sequencing primer (5'-AAGAACATCGATTTTCCATGGCAG-3') were used to

ascertain that the isolated plasmids contain the correct insert.

2.10. DNA Sequencing.

Sequencing of all cloned fragments was done using ABI 310 DNA sequencer (Applied Biosystems, USA) available at Princes Haya Biotechnology Center/Jordan University of Science and Technology, Irbid-Jordan, and the pJET1.2 forward sequencing primer (5' CGACTCACTATAGGGAGAGCGGC 3'), and the pJET1.2 backward sequencing primer (5'-AAGAACATCGATTTTCCATGGCAG-3').

Sequencing results were analyzed using the ChromasPro software 1.34. The nucleotide sequences were analyzed by BLASTed against the GeneBank using "blastn" provided by the National Center for Biotechnology Information (NCBI).

2.11. Phylogenetic Analysis.

The 196 base pairs of the 5'NTR enterovirus sequence from 14 isolates obtained in this study were compared to each other and to 56 reference enterovirus strains from GenBank. All sequences were prepared by EditSeq software (DNAStar version 7.0, Inc., Madison, WI, USA) and aligned using the Clustal W method of the MegaAlign software (DNAStar version 7.0, Inc., Madison, WI, USA). The phylogenetic tree was constructed by the neighbor-joining method and the reliabilities were evaluated with 1000 bootstrap replications.

3. Results

3.1. Qualitative detection of enterovirus in the concentrated samples.

Table 2 summarizes the detection of enterovirus and recovery yield of the bacteriophages. For all sites, the recovery yields of spiked bacteriophage PRD1 represent the mean of at least three different samples. The recovery yields means for the surface water samples were between 5 to 6%. The highest recovery yields were obtained from treated sewage.

Table 2. Detection of enterovirus by RT-PCR and southern hybridization assays in the concentrated surface water and raw sewage samples, and recovery yields of bacteriophage PRD1 from the various samples collected.

Sample	No. of Samples	% Recovery mean ± S.D. [a]	No. (%) of samples positive
Site A	3	2 ± 0.94	3 (100)
Site B	3	8 ± 1.2	1 (33)
Site C	7	6 ± 0.5	2 (29)
Site D	7	5 ± 2.3	3 (42)
Site E	3	6 ± 1.4	1 (33)
Site F	3	8 ± 1.3	0 (0)
Site G	7	5 ± 1.6	4 (57)
Total	33		14 (42)

[a]The mean and standard deviation (SD) obtained from at least 3 different samples

A sample was considered positive for enteroviral RNA if the 196-bp RT-PCR product gave a positive signal after southern blotting hybridization against a specific probe (EV: 5´-ACTACTTTGGGTGTCCGTGTTTC-3´). Of the 33 samples collected, 14 (42%) were found positive for enterovirus RNA (Table 2). The number of surface river water samples tested positive for enteroviral RNA were 1(33%) at site B, 2 (29%) at site C, 3 (42%) at site D, and 4 (57%) at site G. Figure 2 shows an example of enterovirus positive results by RT-PCR and southern blotting hybridization. Enterovirus RNA was detected in only 1 out of 3 (33%) treated sewage samples collected from site E. All raw sewage samples (100%) were contained enteroviral RNA (Table 2).

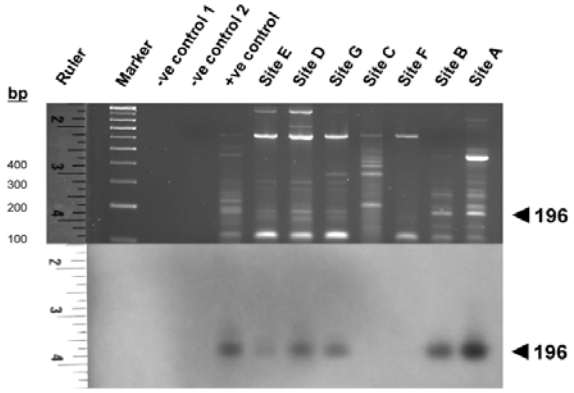

Figure 2. Enterovirus specific RT-PCR products (A) and southern blotting hybridization (B). Ruler: 30 cm long fluorescent ruler; Marker: 100-bp ladder; -ve control 1: negative control for RNA extraction; -ve control 2: negative control for RT-PCR; +ve control: stool sample positive for enterovirus.

3.2. Semi-quantitative estimation of enteroviruses concentration.

The RNA virus concentration of the positive samples ranged from 5 to 10^4 pdu/ml. The highest concentration of enterovirus RNA was in the raw sewage samples with a mean of 2.5×10^3 pdu/ml, whereas the lowest concentration (8.3 pdu/ml) was obtained from the surface river water sample collected from site C (Table 3). Samples collected in March were positively confirmed for enteroviral RNA by RT-PCR and southern blotting hybridization for all sites except for sites C and F (Fig. 2).

Table 3. Enterovirus concentration in all samples collected.

Date of sampling	Concentration (pdu/ml) [a]						
	Site A	Site B	Site C	Site D	Site E	Site F	Site G
28 - Feb	1 X 10^3	0	0	10 *	ND	ND	0
19 - Mar	3.3 X 10^3	6.3 *	8.3 *	10 *	25	ND	10 *
04- June	ND	ND	0	0	ND	ND	0
23 - Aug	ND	ND	0	0	ND	ND	0
19 - Oct	3.3 X 10^3	0	0	0	0	0	26.6
15 - Nov	ND	ND	0	0	ND	0	11.1
06 - Dec	ND	ND	11.1	25	0	0	66.6

[a] pdu/l: PCR detectable units per ml, after detection by RT-PCR and southern hybridization.

* Mean recovery yield was used to calculate concentration.

ND: Not Determined

3.3. Cloning and DNA sequencing

Blast results indicated that one out of the 14 samples contained mixtures of 3 different types of enterovirus

amplicon, two samples contained mixtures of 2 different types of enterovirus amplicons, and 9 samples contained only one type of enterovirus amplicons. Two RT-PCR products failed to be cloned.

The results of sequence analysis of enteroviruses demonstrated that the most frequently detected types were poliovirus 1 followed by coxsackievirus B4; both were detected in 6 samples. Echovirus 11 was detected in 2 samples, while echovirus 9 and coxsackievirus B3 were detected only once (Table 4).

Table 4. Enterovirus serotypes present in the samples collected along Al Zarqa River.

Date of sampling	Virus type [a]						
	Site A	Site B	Site C	Site D	Site E	Site F	Site G
28 - Feb	PV1, CVB 4			ND			
19 - Mar	PV1, CVB 4, E9	ND	CVB 4	PV1	PV1		CVB 4, PV1
04- June							
23 - Aug							
19 - Oct	CVB 4						CVB 4
15 - Nov							CVB 3
06 - Dec			E11	E11			PV1

[a]Virus type was determined by sequence analysis 196-bp RT-PCR products. PV1: Poliovirus 1; CVB: Coxsackievirus B; E: Echovirus.

ND: Not Determined.

3.4. Analysis and comparisons of the obtained sequences against the sequences in databases.

The obtained sequences from all enetroviruse-positive samples were compared to reference strains of human enteroviruses by alignment of the 196-bp RT-PCR product (Fig. 4). Similarities were observed between sequences detected in samples from the same collection site on the same day and also on different days. Coxsackievirus B3 isolate had a similarity of approximately 93% with the reference strain CVB3-Nancy, whereas isolate echoviruses 9 had 90% with E9-Hill. Coxsackievirus B4 isolates detected in samples collected on the same or different days had a similarity range from 86% to 92% with the reference strain CVB4-E2.

A phylogenetic tree based on the similarities of sequences of the 196-RT-PCR products was generated (Fig. 5). All six poliovirus 1 isolates clustered with one another, and with poliovirus 1–Sabin strain. Likewise, coxsackievirus B4 isolates clustered together and with the CVB4-E2 strain. Echovirus 11 isolates tightly clustered with one another, and with E11-Gregory. Both echovirus 9 and coxsackievirus B4 isolates clustered with E9-Hill and CVB3-Nancy, respectively.

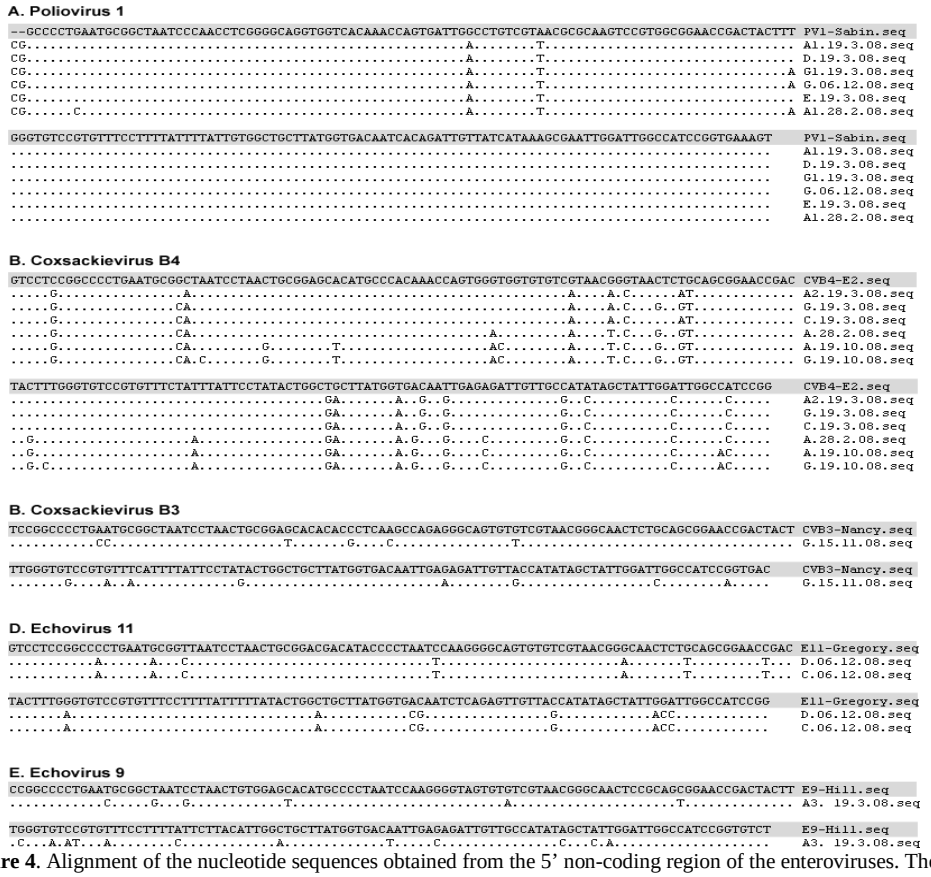

Figure 4. Alignment of the nucleotide sequences obtained from the 5' non-coding region of the enteroviruses. The sequences are designated by the letter of the sampling site and a number matching to the year, the month and the day of sample collection. The shades sequences are for the reference enterovirus strains, which are from top to bottom: Poliovirus 1 Mahoney (Accession no. V01148); coxsackievirus B4 CVB4-E2 (Accession no. S76772); coxsackievirus B3 CVB3-Nancy (Accession no. M16572); Echovirus 11 E11-Gregory (Accession no. X80059); Echovirus 9 E11-Hill (Accession no. X84981).

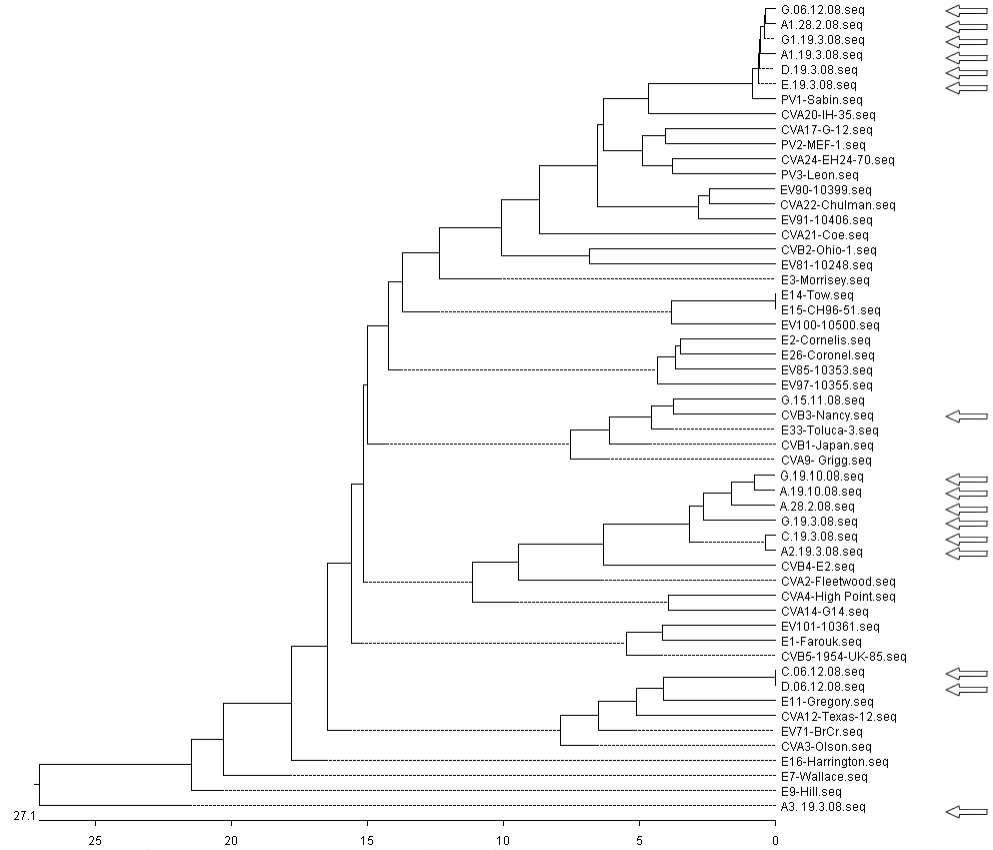

Figure 5. Phylogenetic analysis of enterovirus sequences obtained from different samples. The scale represents the number of nucleotide substitution between the sequences. The arrows locate the enteroviral sequences identified in this study.

4. Discussion

This study is focused primarily on enteroviruses in water environmental samples collected from Al-Zarqa River, Jordan as literature review showed the absence of any qualitative and/or quantitative information about those viruses in the country.

4.1. Concentration of water samples.

It is worth mentioning that the recovery yields of enteroviruses were higher for the less turbid samples (e.g. treated water), compared with more turbid ones (e.g. raw sewage). Similarly, the closer the site of collection to ASWWTP, the better the recovery yield obtained. This observation is consistent with other studies. Katayama *et al.* (2008) obtained recovery yields of 28% and 65% for sewage and treated water, respectively. The possible explanation for the low recovery yields of enteroviruses as well as the bacteriophage PRD1 associated with turbid samples may be the adsorption of the virus into the particles present in the mixture.

4.2. Qualitative and semi-quantitative estimation of enteroviruses.

The annealing temperature of the primers used in enterovirses PCR protocol was 37°C, which is considered to be low. With this low annealing temperature, there is a high probability of non-specific binding of the primers, which in turn will give a false positive result that necessitates the addition of a confirmatory step. Therefore, southern blotting hybridization against an enteroviral specific probe was used. A sample was only considered positive for enteroviral RNA by southern blotting if the 196-bp RT-PCR product gave a positive signal.

Of the 33 samples collected, 14 (42%) tested positive for enterovirus RNA. This frequency is similar to that of other studies (Chapron *et al.*, 2002; Lee and Kim, 2002). With the exception of pond water samples (Site F), the presence of enterovirus RNA was detected in all sites. All the collected raw sewage samples also contained enteroviral RNA, which is consistent with other studies (Green and Lewis, 1999; Lodder and de Roda Husman, 2005; Katayama *et al.*, 2008) that showed the raw sewage usually contains high viral loads. The mean concentration of enteroviral RNA in raw sewage samples was 2.5 X 10^3 pdu/ml. The number of enterovirus pdu present in all samples was estimated semi-quantitatively by RT-PCR and southern blotting hybridization on tenfold serially diluted RNA. Because it detects the enteroviral RNA molecules, the concentration of enteroviral RNA expressed as PCR-detectable unit (pdu) does not usually reflect the concentration of infectious enteroviruses particles, in contrast to the plaque forming unit. Therefore, enterovirus concentrations may be even lower than the obtained result. However, this unit might be beneficial when a comparison between samples is required, especially between raw sewage and treated water samples. Because we were unable to obtain the Buffalo Green Monkey Kidney cell line that usually used to grow enteroviruses, semi-quantitave estimation using the pdu method was the only way to compare samples with respect to enterovirus concentration. Comparisons of enterovirus concentration

obtained from our samples with those of other studies cannot be made, since all studies use the plaque forming unit to express the concentration of enteroviruses.

Only one treated water sample collected from the ASWWTP effluent gave a positive result for enteroviruses RNA. By comparing between the samples collected from the inlet and outlet of ASWWTP, 2.7 log^{10}-unit of enterovirus removal was observed when samples collected on March 19th. This implies that the treated sewage water contains 5 enterovirus pdu discharged into Al-Zarqa River surface water.

Out of the 21 samples of river surface water collected, fifty seven percent tested positive for enteroviral RNA (Table 4). The highest number of samples tested positive for enteroviruses were in site G (4 samples), followed by site D (3) then site C (2). It was noted that as we went downstream ASWWTP, the number of enterovirus-positive samples increased. This can be explained by the fact that, both human (e.g. farming and industrialization) and environmental factors affecting the quality of the river are gradually amplified as the river reaches its final destination in King Talal Dam.

Only one sample collected from site E contained enterovirus RNA. Therefore, untreated water coming from Al-Zarqa city has adverse effects on the water quality of Al-Zarqa River. All samples collected from the private pond were enterovirus RNA free. The pond is fed from groundwater; therefore, filtration of viruses might have occurred during water passage through the soil (Saadoun *et al.*, 2008) resulting in viruses free filtered water.

None of the samples collected in July and August contained enterovirus RNA. In Jordan, the temperature is higher during this period than the rest of the months during which the samples were collected. This observation contradicts other reports showing a peak of enterovirus circulation in the summer and early autumn (Muir *et al.* 1998; Katayama *et al.*, 2002; CDC, 2006; Jiang *et al.*, 2007). However, this may be explained by knowing that the high number of enteroviruses infections comes from using water resources for recreation, which is not the case for Al-Zarqa River that not used for recreation. Furthermore, enteroviruses survive longer at lower temperatures in natural environments, such as river and groundwater (Fong and Lipp, 2005). Although enteroviruses were detected in clinical samples (Meqdam *et al.*, 1997a, 1997b) and house hold water samples (Malkawi and Shaban, 2007), there were no studies in Jordan testing the presence of enteroviruses in river water or water basin to conduct a comparison.

4.3. Typing of enteroviruses detected by sequence analysis.

As cloning was used to test the co-presence of multiple enterovirus serotypes in single samples, we demonstrated the simultaneous presence of 3 different serotypes of enteroviruses in one sample and 2 serotypes of enteroviruses in two different samples. Cloning of virus-positive PCR product has been used extensively to test for the co-presence of multiple virus types in different water environmental samples (Lee and Kim, 2002), especially raw sewage samples, where it is suspected to harbor several serotypes (van den Berg *et al.*, 2005).

Sixteen sequences were obtained from cloning the 14 enterovirus positive RT-PCR products. To identify the serotype of enterovirus present in each sample, the enterovirus sequences were BLASTed against the GeneBank databases using " blastn" provided by the National Center for Biotechnology Information (NCBI). The BLAST reports obtained demonstrated the presence of both poliovirus type 1 and coxsackievirus B4 in 6 samples for each, Echovirus 11 in two samples, and coxsackievirus B4 and Echovirus 9 in one sample for each.

4.4. Phylogenetic analysis.

Two echovirus 11 isolates were identified in two different surface river water samples collected from sites C and D in December 6th. Alignment and phylogentic analysis of the two isolates showed that they were identical and related to echovirus 9 Hill strain. The occurrence of these two identical isolates indicates that they came from the same origin. ASWWTP outlet may be the source of echovirus 11 identified in sites C and D. However, this relationship cannot be ascertained, because sewage and treated water samples were not collected from the inlet and outlet of ASWWTP on the same day. The absence of this serotype in site G is due to either dilution of this serotype along the river and/or its inactivation.

The six coxsackievirus B4 isolates obtained were closely related and clustered together with the CVB4-E2 reference strain. By comparing them against each other, the three isolates collected in March 19th were closely related to each other more than to other isolates collected on different dates. This indicates that they may have originated from the same source, ASWWTP inlet. Supporting this presumption is the high similarity of coxsackievirus B4 sequences isolated from the inlet of ASWWTP, 2 and 40 km downstream ASWWTP at sites C and G, respectively. This trend is also strongly supported by 4 poliovirus 1 isolates identified in the same collection day and from overlapping sites (A and G).

5. Conclusion.

This study demonstrated the circulation of enterovirus in large volumes of sewage and water environmental samples collected from and around Al-Zarqa River. Enterovirus serotyping and phylogenetic analysis enabled us to trace back the source of viral contamination in some samples.

Acknowledgments.

The Deanship of Scientific Research at Jordan University of Science and Technology, Irbid-Jordan (Grant No. 58/2008) thankfully funded this study. Authors would like to express their deep thanks to National Institute of Public Health and the Environment (RIVM), the Netherlands, for their technical training and advice, to engineer Sufyan Bataineh from Water Authority of Jordan for his help in collecting samples from ASWWTP, and to Dr. Mustafa Ababneh/JUST-Jordan for providing the DNAStar software. Appreciation is extended to University of Sharjah/Sharjah-UAE for the administrative support.

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