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Genomic detection of waterborne enteric viruses as water quality indicators in Al-Zarqa River, Jordan

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ABSTRACT

Al-Zarqa River is the second main tributary to River Jordan after the Yarmouk River. The river flow has been modified by discharge of industrial wastewater and treated domestic water. Concerns about the occurrence of waterborne pathogenic viruses in the surface waters of Al-Zarqa River prompted the analysis of the surface water quality with respect to the presence of enteric viruses. Viruses were concentrated from a total of 33 different water environmental samples including raw sewage, effluent samples and river water collected from and around the river over a period of 11 months. Calculated recovery yields for these concentration methods ranged between 2 and 8%. Polymerase chain reaction (PCR), reverse transcriptase-PCR (RT-PCR), nested RT-PCR and southern blotting hybridization analysis were used for the detection of hepatitis A virus, norovirus, astrovirus and human adenovirus 40/41, with the later one being detected in 21 (64%) of the samples that also showed previous positive presence for enteroviruses. To our knowledge, this is the first molecular biology report in Jordan describing the circulation of adenoviruses, which were detected more frequently than enteroviruses in sewage and water samples, and therefore, they can be used as an index for the presence of human pathogenic viruses in water environment.

Key words: adenoviruses, astroviruses, enteric, hepatitis A viruses, wastewater, Zarqa River

HIGHLIGHTS

- Occurrence of waterborne pathogenic viruses in water resources.
- Circulation of adenoviruses from and around Al-Zarqa River, Jordan.
- Implementation of techniques for concentrating viruses from surface and treated water, and raw sewage.
- Genomic content of enteric viruses was detected by molecular techniques.
- Adenoviruses can be used as an index for the presence of human pathogenic viruses in water environment.

LIST OF ABBREVIATIONS

ASWWWTP	As-Samra Wastewater Treatment Plant
EPA	Environmental Protection Agency
PCR	Polymerase chain reaction
PRD1	Bacteriophage PRD1 (Somatic salmonella phage (SOMSPH) based on ISO 10705-1)
RT-PCR	Reverse transcriptase-polymerase chain reaction
RIVM	The National Institute of Public Health and the Environment

INTRODUCTION

Jordan is classified as water scarce with aquifers and basins, which represent Jordan's primary sources of water. The Yarmouk Basin is the largest in the country followed by the Amman-Zarqa basin. The area of Amman-Zarqa basin is located in the most densely populated area in Jordan, accounting for 65% of its population, 90% of the industries in the country, and provides irrigation for 8,400 ha of land around (Arab Environment Monitor (AEM) 2006).

The industries in the city of Zarqa produce wastewater of low to high pollution loads. Most of these industries are linked to the public sanitary network, where domestic wastewater containing high organic loads from about 2 million inhabitants in Jordan flows into the As-Samra Wastewater Treatment Plant (ASWWTP). Al Zboon & Al Suhaili (2009) reported that the BOD and COD concentration of effluent from wastewater treatment plant (As-Samra) exceeded the allowable Jordanian standards, which indicates a high load of pollution. In addition, ASWWTP discharges about 110 MCM of partially treated wastewater and this amount is considered a source of high pollution, especially years after the start of the wastewater treatment, as the efficiency of the treatment plant has decreased (Al Manaseer et al. 2020).

The lower effluent quality from ASWWTP is expected due to long periods of high load to a level with substantial loads of potentially pathogenic microorganisms to remain. This will lead to infiltration of wastewater from ASWWTP and thus the groundwater quality in the Zarqa basin is deteriorated, which will undermine the quality of life of residential communities in the Amman-Zarqa basin (Westrell 2004).

In some places around the world, crops are irrigated with wastewater, and the transmission of pathogens to humans (and animals) can occur directly or via crops, aerosols and potentially via groundwater. The transmission of pathogens via the environment can clearly result in infections and diseases, and secondary person-to-person transmission can occur. In this way, the circulation of pathogens in the environment will continue (Westrell 2004).

The pathogens of main concern in the Al-Zarqa River, one of the major water resources in Jordan, include bacteria, protozoa and viruses. The last type of microorganisms are of main concern as they generally pose a greater challenge than bacteria and protozoa in food and wastewater treatment and the risk of infection can be 10–1,000 times higher than for bacteria at a similar level of exposure (Rose & Gerba 1991). Also, viruses can survive in water longer than bacteria and can tolerate changes in temperature and pH (Maunula et al. 2005). In addition, viruses are more resistant to commonly employed water and wastewater disinfection. Therefore, viruses can easily be harbored in 'microbiologically immaculate' water (Payment et al. 1994; Lees 2002).

Waterborne viruses belong to the large group of enteric viruses that are shed in feces. The enteric viruses described in this study infect and may cause disease in humans (Fong & Lipp 2005).

The majority of the human enteric viruses belong to the virus families *Caliciviridae* (e.g. norovirus), the *Astroviridae* (e.g. astroviruses) and *Reoviridae* (e.g. reo- and rotaviruses), as well as some members of the *Adenoviridae* (e.g. adenovirus 40/41). Enteric viral gastroenteritis is probably the most common disease responsible for diarrhea worldwide (Lopman et al. 2004). Infection with adenoviruses may also lead to gastroenteritis and alternatively to conjunctivitis and/or pharyngitis (Fong & Lipp 2005). Adenoviruses are included in the 'Candidate Contaminant List' generated as part of the Safe Drinking Water Act by the U.S. Environmental Protection Agency (EPA), and this list includes also caliciviruses (e.g. norovirus), hepatitis A and some enteroviruses (namely coxsackieviruses and echoviruses) (EPA CCL3 2009).

Studies in Jordan about the occurrence of waterborne pathogenic viruses in water resources are still in their early stages. Many of the monitoring programs and research goals are focusing on bacteria and protozoa as the microbiological parameters of water quality, and there are a few numbers of reports on the virus level in the water environment in Jordan. For example, only one study performed by Shaaban & Malkawi (2007) tested the presence of viruses in water and wastewater environmental samples, collected from different resources in Jordan. Another study by Saadoun et al. (2008) evaluated the performance of riverbank filtration technology aside from Al-Zarqa River to reduce fecal coliform indicators and bacteriophages. Recently, Saadoun et al. (2020) described the surface water quality of Al-Zarqa River with respect to the presence of enteroviruses, with a special focus on their serotypes and phylogeny. The limited data on viral occurrence in water in Jordan prompted us to investigate the surface water quality of Al-Zarqa River with respect to the presence of waterborne human pathogenic viruses, especially the enteroviruses and enteric adenovirus 40/41. Therefore, in this study, the presence of adenovirus 40/41, hepatitis A virus, norovirus and astrovirus in raw sewage, treated water and surface water was evaluated by detecting the genomic content of the above viruses by reverse transcriptase-polymerase chain reaction (RT-PCR), nested RT-PCR and southern hybridization.

MATERIALS AND METHODS

Site location, collection of surface water, pond water, raw and treated sewage samples

Sites of collection along the river as well as their conditions are the same as reported recently by Saadoun et al. (2020). Figure 1 presents a summary of the samples and the map shows physically the places along the river where the samples
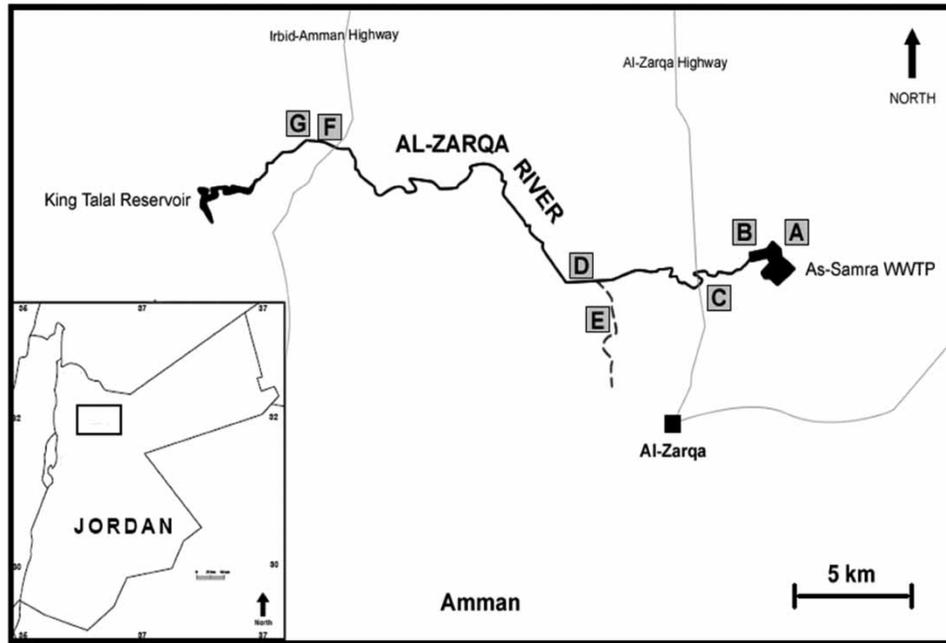
Figure 1 | Locations of sampling sites along Al-Zarqa River. A: ASWWTP inlet; B: ASWWTP outlet; C: 2 km downstream the ASWWTP, Al-Hashemia city; D: 10 km downstream the ASWWTP, Al-Sukhna town; E: Al-Sukhna town bridge; F: 40 km downstream the ASWWTP, Jerash bridge, Jordan Modern Private Nursery; G: 40 km downstream the ASWWTP, Jerash bridge, Jordan Modern Private Nursery (Saadoun *et al.* 2020).

were collected. A total of 33 samples of raw sewage, treated sewage, surface and pond water were collected from various places along Al-Zarqa River at different distances (Table 1); 2, 11 and 40 km downstream from ASWWTP effluent over a period of 1 year following the same scheme were reported by Saadoun *et al.* (2020) to study the types and quantities of enteric viruses. Samples were kept cool and processed as soon as possible or stored at −70 °C until processed.

A major intake for Al-Zarqa River flow, especially in the summer, is the ASWWTP effluent. Therefore, two composite samples collected over 24 h were taken from the inlet and outlet of the treatment plant. The inlet samples contain raw sewage and thus are expected to contain high viral loads; therefore, only 1 l of these samples was collected. In contrast, 2 l were collected from the outlet, which discharge treated water, and the samples collected from this site could give a good idea on the performance of ASWWTP in the removal and/or inactivation of viruses by the current applied treatment processes. Because the concentration of waterborne viruses present in the river might be low, a large volume of 7–8 l of surface water was collected from the 2, 11 and 40 km downstream ASWWTP.

Table 1 | The location, type, number and the volume of the samples collected along Al-Zarqa River (Saadoun *et al.* 2020)

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

The 2 km site was chosen because it is located near both the ASWWTP outlet and the first animal and crops farm residing along the river. Therefore, surface water samples collected from this site could be used to test the possibility that these farms might affect the quality of the previously treated water in the ASWWTP.

The 11 km site is located near Al-Sukhna town, where the magnitude of both industrial and agricultural activities affecting the river quality increases. In addition, a flow of treated water coming from Al-Zarqa city meets with the river at this site; therefore, the contribution of this flow to Al-Zarqa River with respect to water quality was estimated, by collecting water samples from both sites and from the treated water coming from Al-Zarqa city, at an upstream location that meets with Al-Zarqa River by 5 km.

Finally, the 40 km site was chosen for two reasons: first, it is only 2 km upstream from King Talal Dam, which is the final destination for Al-Zarqa River, therefore, providing information about the viral water quality in the dam. The second reason is because it is one of the farthest points downstream ASWWTP at which, the combined factors affecting the quality of the river can be studied extensively, such as the effect of both human (e.g. farming and industrialization) and environmental factors.

A limited number of 2 l samples were collected from an artificial pond at Jordan Modern Nursery (Site G). These samples mimic to some extent the groundwater in the Al-Zarqa basin. Testing these samples for the occurrence of viruses might give an idea about the infiltration of these viruses into the groundwater resources.

Concentration of surface water and raw sewage samples, and calculation of virus recovery

Surface water and raw sewage samples were concentrated similarly as described by Saadoun et al. (2020). Surface water samples were concentrated by the adsorption–elution method (Haramoto et al. 2005) with modifications as described by Saadoun et al. (2020). 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.

The volume of all water samples was reduced to 10 ml, and the concentration factor ranged from 100 to 750. The 1 l raw sewage samples collected in this study were concentrated 100 times, a concentration factor that is comparable with other studies (Formiga-Cruz et al. 2005; van den Berg et al. 2005).

The recovery percentage of the applied concentration methods used in this study was calculated similarly as described by Saadoun et al. (2020). In brief, the collected water and sewage samples were spiked 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 unconcentrated 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:

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

RNA and DNA extraction from retentate

Viral RNA and DNA were extracted from retentates using the silica beads in the presence of guanidinium isothiocyanate (Fluka, Switzerland) following the method developed by Boom et al. (1990) and as described previously by Saadoun et al. (2020). One hundred microliters of retentate was mixed with 900 μl lysis buffer L6 and 40 μl silica suspension and incubated for 10 min. After that, the mixture was centrifuged for seconds at 12,000 × g (Hettich, Germany), and the silica pellet was washed twice with 1 ml washing buffer L2, 70% ethanol (v/v) (GCC, UK) and finally once with acetone (Frutarom, UK). Subsequently, the silica pellet was dried at 56 °C for 10 min in water bath (GFL, Germany), then resuspended in 50 μl of elution buffer, vortexed briefly and incubated at 56 °C for 10 min. Next, the silica was briefly vortexed again and centrifuged for 2 min at 12,000 × g, and the supernatant containing RNA or DNA was stored at −70 °C until use.

RT-PCR amplification and gel electrophoresis

Primers and probes used in RT-PCR, PCR and southern hybridization assays for the detection of adenovirus (40/41), norovirus, astrovirus and hepatitis A virus are listed in Table 2. PCR products were separated onto 2% agarose (Biobasic, Canada) in the presence of 1 μg/ml ethidium bromide (Promega, USA).

Table 2 | Primers and probes used in RT-PCR, PCR and southern hybridization assays for the detection of adenovirus, norovirus, astrovirus and hepatitis A virus

Virus	Primer/probe	Sequence (5′ … 3′)	Product size (bp)	References
Norovirus	JV13i	TCATCATCACCATAGAAIGAG	326	Vennema et al. (2002); Saskia et al. (2006)
	JV12y	ATACCACTATGATGCAGAYTA		
	GGI (probe)	ATGGAYGTTGGYGAYTATGT		
	GGII (probe)	GAAYTCCATCRCCCAYTG		
	UK3 (probe)	GTCCCCTGACATCATACAGGCT		
	JV5 (probe)	CTCACCAGAGGTTGTCCAAGC		
Adenovirus (40/41)	hexAA1885	GCCGCAGTGGTCTTACATGCACATC	301	Formiga-Cruz et al. (2005)
	hexAA1913	CAGCACGCCGCGGATGTCAAAGT		
	hexAA1893	GCCACCGAGACGTACTTCAGCCTG	143	
	hexAA1905	TTGTACGAGTACGCGGTATCCTCGCGGTC		
Hepatitis A	HAV1	TTGGAACGTCACCTTGCAGTG	368	Formiga-Cruz et al. (2005)
	HAV2	CTGAGTACCTCAGAGGCAAAC		
	neHAV1	ATCTCTTTGATCTTCCACAAG	290	
	neHAV2	GAACAGTCCAGCTGTCAATGG		
Astrovirus	Ast1	GTAAGATTCCCAGATTGGT	243	Chapron et al. (2000)
	Ast2	CCTGCCCCGAGAACAACCAAG		
	A1	CCTTGCCCCGAGCCAGAA	143	
	A2	TATTCACAAACTTATGGCAA		

RT-PCR amplification for norovirus

An 18 μl anti-sense mix composed of 15 μl of extracted RNA plus 100 pmol of the reverse primer JV13i 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 1× PCR buffer (10 mM Tris–HCl [pH 8.3]), 3 mM $MgCl_2$, 1 mM dNTPs 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 chilled on ice for at least 5 min. PCR was performed by adding 15 μl of cDNA, the forward primer JV12y, 1× PCR buffer (10 mM Tris–HCl [pH 8.3]), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 5 U of Taq Polymerase (all from Promega, USA). PCR cycling was as follows: initial denaturation for 3 min at 94 °C, 45 cycles 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.

NESTED PCR AMPLIFICATION FOR ADENOVIRUS 40/41

Ten microliters of extracted DNA was added to 40 μl of PCR mixture in a final concentration of 1× PCR master mix (Promega, USA) and 0.4 μM from the reverse (hexAA1885) and forward primers (hexAA1913) (Table 2). The conditions during the 30 cycles of the amplification were denaturing at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. For the nested PCR amplification, 1 μl of the amplified DNA from the first PCR was added to a new batch of 49 μl of PCR mix containing 3.5 pmol of adenovirus inner primers (hexAA1893) and (hexAA1905) (Table 2). The amplification cycles were as described previously.

Nested RT-PCR amplification for astrovirus and hepatitis A virus

The amplification conditions of the nested RT-PCR methods used for detecting astrovirus and hepatitis A virus were the same. A 7 μl anti-sense mix composed of 5 μl of extracted RNA plus 20 pmol of the reverse primers (astrovirus, Ast2; hepatitis A virus, HAV2) 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 3 μl reverse transcription mix containing 1× PCR buffer (10 mM Tris–HCl [pH 8.3]), 3 mM $MgCl_2$, 1 mM dNTPs 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 chilled on ice for at least 5 min. PCR was performed by adding 15 μl of cDNA, 0.4 μM of the forward and reverse primers (astrovirus, Ast1 and Ast2; hepatitis

A virus, HAV1 and HAV2), 1× PCR buffer (10 mM Tris–HCl [pH 8.3]), 1.5 mM MgCl$_2$, 0.2 mM dNTPs and 5 U of Taq Polymerase (all from Promega, USA). PCR cycling was as follows: initial denaturation for 3 min at 95 °C, 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C followed by a final extension step for 10 min at 72 °C. For the nested PCR amplification, 1 μl of the amplified DNA from the first PCR was added to a new batch of 49 μl of PCR mix containing 3.5 pmol of astrovirus and hepatitis A virus inner primers (astrovirus, A1 and A2; hepatitis A virus, neHAV1 and neHAV2) (Table 2). The amplification cycles were as described previously.

Gel electrophoreses

All PCR products were separated on 2% agarose (Biobasic, Canada) gels with a concentration of 1 μg/ml ethidium bromide (Promega, USA) at 120 volt and visualized using the Vilber Lourmat UV detection system (Japan).

Extraction of PCR products from gels

PCR products from gels were extracted as described before by Saadoun et al. (2020).

Southern blotting hybridization

Agarose gels containing the RT-PCR products were similarly hybridized as described by Lodder & Husman (2005) and reported by Saadoun et al. (2020). The probes used for southern hybridization assays for the detection of norovirus are listed in Table 2. Detection was made using biotin chromogenic detection kit (Fermentas, EU) following the manufacturer's instructions.

Quality control of the amplification method

Standard precautions were applied in all manipulations to reduce the possibility of sample contamination by amplified DNA molecules. Separate laboratories/working areas and micropipette sets were used for virus concentration, RNA extraction and preparation of RT-PCR mixtures. All reagents used in RNA extraction and RT-PCR were DNAse-RNAse free. In addition, micropipette tips used were all filtered. Negative controls for RNA extraction, RT-PCR and nested PCR were included in each test.

RESULTS

Samples collection

A total of 33 different types of samples were collected from and around Al-Zarqa River and ASWWTP (Table 1). Three of each of composite raw (Site A) and treated sewage (Site B) samples were generously provided by the ASWWTP staff. These samples were collected over 24 h. Other treated wastewater samples were collected from Al-Sukhna town (Site E). Those samples originate from the wastewater treatment plant in Al-Zarqa city. Twenty-one surface water samples (Sites C, D and G) were collected at different river distances (2, 11 and 40 km) along the river and downstream ASWWTP. Three pond water samples were collected from 'Jordan Modern Nursery' located next to Jerash Bridge, Irbid-Amman highway.

Concentration of samples and recovery of spiked bacteriophage PRD1

Table 3 shows the detection of adenovirus 40/41 and other enteric viruses by PCR, 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 at each site along Al-Zarqa River.

The recovery yields of bacteriophages PRD1 spiked into the collected samples are summarized in Table 3. For all sites, the recovery yields of spiked bacteriophage PRD1 represent the mean of at least three different samples. The recovery yield means for the surface water samples were between 5 and 6%. The highest recovery yields were obtained from treated sewage, with a mean of 6% at site F and 8% at site B, and pond water samples, a mean of 8%. Only 2% of spiked bacteriophage PRD1 was recovered from raw sewage samples, which was the lowest recovery yield mean obtained from all samples.

Presence of enteric viruses

The presence of adenovirus 40/41, norovirus, hepatitis A virus and astrovirus genomes in the collected samples was investigated. Norovirus RNA was detected by means of RT-PCR followed by southern blotting hybridization, whereas for adenovirus 40/41, hepatitis A virus and astrovirus, nested RT-PCR protocols were carried out to detect them.

Table 3 | Detection of adenovirus 40/41 and other enteric viruses by PCR, RT-PCR and southern hybridization assays in the concentrated surface water and raw sewage samples, and recovery yields of bacteriophage PRD1[a] from the various samples collected

Sample[b]	No. of samples	% Recovery mean ± SD[c]	No. of samples positive (%)	
			Adenovirus 40/41[d]	Other enteric viruses[e]
Site A	3	2 ± 0.94	3 (100)	0 (0)
Site B	3	8 ± 1.2	1 (33)	0 (0)
Site C	7	6 ± 0.5	4 (57)	0 (0)
Site D	7	5 ± 2.3	5 (71)	0 (0)
Site E	3	6 ± 1.4	2 (66)	0 (0)
Site F	3	8 ± 1.3	1 (33)	0 (0)
Site G	7	5 ± 1.6	5 (71)	0 (0)
Total	33		21 (64)	0 (0)

[a]PRD1: bacteriophage PRD1 [Somatic salmonella phage (SOMSPH) based on ISO 10705-1] [ISO 10705-1:1995 (E)].
[b]Site location, designation and conditions of each sample were as described previously by Saadoun et al. (2020).
[c]The mean and standard deviation (SD) obtained from at least three different samples.
[d]Detection by nested PCR.
[e]Norovirus, detected by RT-PCR followed by southern blotting hybridization. Both hepatitis A virus and astrovirus were detected by nested PCR.

A positive nested PCR detection assay of the adenovirus 40/41 genome was demonstrated in 21 (64%) of the 33 concentrated samples tested (Table 3). The number of samples testing positive for adenovirus genome was one (33%) at sites B and F, two (66%) at site E and four (57%) for site C. The highest number of positive samples was in sites D and G, with five (57%) samples positive for adenovirus 40/41 genome in each site. All raw sewage samples (100%) contained adenovirus 40/41 genome (Figure 2). Moreover, enteroviruses that were previously reported by Saadoun et al. (2020) to be positive in 13 out of 14 samples were also positive for human adenoviruses in samples collected in February from sites A and D; in March from sites A, B, C, D and E; in October from sites A and G; in November from site G; and in December from sites C, D and G (Table 4).

Norovirus RNA was not detected in all 33 collected samples by means of RT-PCR and southern blotting hybridization. To ensure that the norovirus-specific primers were not inhibited in the RT-PCRs, the extracted RNA from a stool sample, previously known to be positive for norovirus, was mixed with the extracted RNA from a surface water sample with a ratio of 1:5. Both the mixed RNA and the stool sample extracted RNA tested positive for norovirus RNA, in contrast to the RNA of collected samples that tested negative (Figure 3).

Hepatitis A virus and astrovirus genomes were not detected in all the 33 collected samples by means of nested RT-PCR. To test the possibility that the negative results obtained for these two viruses were because of some sort of error during the RT-
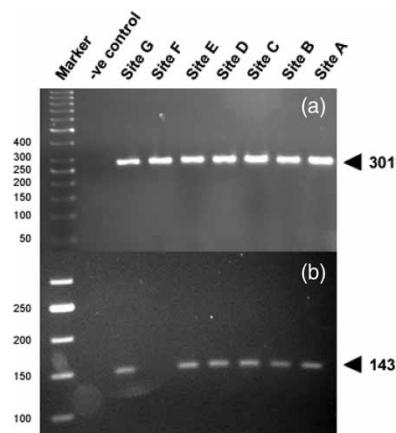

Figure 2 | Adenovirus 40/41 PCR products visualized by fluorescent agarose gel electrophoresis. (a) First-round PCR. (b) Nested PCR. Marker, 50-bp ladder; −ve control, negative control for PCR and nested-PCR.

Table 4 | Cumulative results for adenoviruses in all samples

Sampling date	Sampling site	Detection results Adenovirus 40/41
28 Feb.	A	+[a]
	B	−
	C	+
	D	+[a]
	G	+
19 Mar.	A	+[a]
	B	+[a]
	C	+[a]
	D	+[a]
	E	+[a]
	G	−
04 Jun.	C	+
	D	−
	G	−
23 Aug.	C	−
	D	−
	G	+
19 Oct.	A	+[a]
	B	−
	C	−
	D	+
	E	−
	F	−
	G	+[a]
15 Nov.	C	−
	D	+
	F	+
	G	+[a]
06 Dec.	C	+[a]
	D	+
	E	+[a]
	F	−
	G	+[a]
Total		21

[a]Samples were also positive for enteroviruses as previously reported by Saadoun et al. (2020).

PCR, a different set of primers, RT-PCR and reagents/kits were used to detect them. However, none of the samples collected tested positive for the genome of these two viruses.

DISCUSSION

The water quality of Al-Zarqa River, Jordan was investigated because it is used mainly for irrigation of 8,400 ha of land around it (AEM 2006). However, to assess the microbiological quality of the river, which usually includes only bacterial
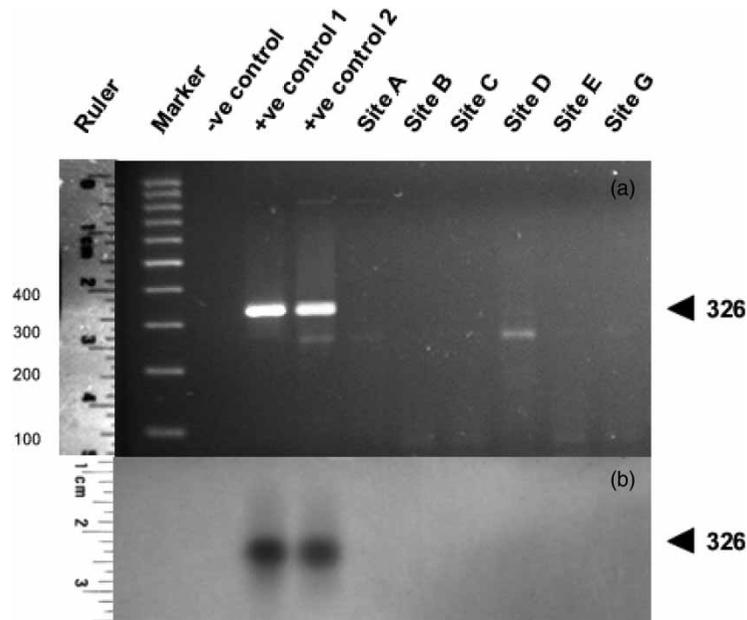
Figure 3 | Norovirus-specific RT-PCR products visualized by fluorescent agarose gel electrophoresis (a) and southern blotting hybridization (b). Ruler: 30-cm fluorescent ordinary ruler; Marker: 100-bp ladder; −ve control: negative control for RT-PCR; +ve control 1: stool sample positive for enterovirus; +ve control 2: mixed RNA from the stool sample and site A sample (ratio 1:5).

fecal indicators, human pathogenic viruses are not included in the regular monitoring programs. The presence of these bacterial fecal indicators may not be correlated with the presence of waterborne viruses (Hot *et al.* 2003), and this was proved by many water outbreaks related to potable water that met all bacteriological standards (Bosch *et al.* 1991; CDC 2006). Therefore, waterborne viruses and their behavior in water attracted the attention of public and water authorities to improve the use and treatment of water and public health as well.

Sample collection

Details about sites of collection along the river as well as their conditions are reported before in the 'Materials and methods' section as well as reported recently by Saadoun *et al.* (2020).

Concentration of samples

Because raw sewage is usually heavily loaded with viruses, some studies use only a few to a few hundred milliliters only as the initial volume to be concentrated (Formiga-Cruz *et al.* 2005; Lodder & de Roda Husman 2005; van den Berg *et al.* 2005), in contrast to the 1 l sewage samples used in this study.

By using the adsorption–elution method, surface, pond and treated water samples were concentrated 750 and 200 times, respectively. Many studies have detected enteroviruses and other viruses in surface river samples concentrated less than 500-fold (Haramoto *et al.* 2005; Westrell *et al.* 2006).

The efficiency of the applied concentration methods was evaluated by spiking the collected samples with a known concentration of bacteriophage PRD1. The percentage of recovery of this bacteriophage from the surface water samples ranged from 5 to 8%, whereas for the raw sewage, it was 2%. In general, the obtained recovery yields are low compared with other studies (Katayama *et al.* 2008; Haramoto *et al.* 2005), where recovery yields of more than 50% were achieved using the same or different concentration methods. The low recovery yield might be explained by the eluant used, skimmed milk compared with NaOH in the above-mentioned studies. Other possible factors include the samples temperature during transportation and the speed of processing.

It is worthy of note that the recovery yields were higher for the less turbid samples (e.g. treated water), compared with more turbid ones (e.g. raw sewage). With respect to surface river water, the closer the site of collection to ASWWTP, the better the recovery yield obtained. This observation is consistent with other studies, for example, 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 for enteric viruses associated with turbid samples is the adsorption of the virus into the particles present in the mixture. This was also noted by Saadoun et al. (2020) for low recovery yields of enteroviruses as well as the bacteriophage PRD1.

Qualitative detection of enteric virus genomes in concentrated samples

The presence or absence of enteroviruses in the environment is not associated with the presence or absence of other important pathogenic viruses (Pina et al. 1998). Therefore, the presence of adenovirus 40/41, hepatitis A virus, norovirus and astrovirus in all collected samples was investigated. These viruses were chosen because they, along with enteroviruses, are the most meaningful, reliable and effective virus index for environmental monitoring of water resources (Jiang & Chu 2004). Moreover, the U.S. EPA included these viruses in their 'Candidate Contaminant List' (Fong & Lipp 2005).

The detection method used for adenovirus 40/41, hepatitis A virus and astrovirus was the nested PCR with two sets of specific primers for each virus type, whereas noroviruses with RT-PCR and southern blotting hybridization following the same approach used by Saadoun et al. (2020) to detect enteroviruses. Because the detection of these viruses is not the scope of this study, a simple, rapid and highly specific method was chosen. On the other hand, the genetic diversity of norovirus is very high; therefore, for the same reason discussed for enteroviruses (Saadoun et al. 2020), RT-PCR followed by southern blotting hybridization, as a confirmatory step, was adapted (van den Berg et al. 2005).

Approximately 64% of the sites were positive for enteric adenoviruses 40/41. All raw sewage samples tested positive for these viruses, this trend was also reported in earlier studies. For example, Katayama et al. (2008) detected adenoviruses in 71 out of 72 (99%) and 71 out of 71 (100%) of raw sewage samples collected from two different wastewater treatment plants. Others detected them in 90% of sewage samples tested (Pina et al. 1998; Formiga-Cruz et al. 2005).

Adenoviruses 40/41 were detected in 21 out of 33 (64%) river surface water samples. Similar frequency (65%) was obtained in another study carried out for river surface water samples (Pina et al. 1998). Like enteroviruses detected by Saadoun et al. (2020), the farther the collection site downstream from ASWWTP, the higher the number of positive samples for adenoviruses 40/41. Saadoun et al. (2020) previously explained this trend 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.

Unlike enteroviruses, adenoviruses 40/41 were detected in one of the three samples collected from the private water pond, and also in one of the three samples collected from the stream coming from Al-Zarqa city (Saadoun et al. 2020). In this study, adenoviruses were more frequently detected than enteroviruses, as 64% of samples were positive for adenoviruses compared with 42% for enteroviruses (Saadoun et al. 2020). This observation is shared by many other studies (Pina et al. 1998; Formiga-Cruz et al. 2005; Rajal et al. 2007). Moreover, approximately 93% of the samples that were positive for enteroviruses (Saadoun et al. 2020) were also positive for human adenoviruses. Therefore, adenoviruses detection by PCR might be used as an index for the presence of pathogenic human viruses in the environment.

In our study, none of the 33 samples collected were tested positive for the presence of hepatitis A virus, norovirus and astrovirus genomes. These viruses might have been present at very low concentrations to an extent that made them undetectable by the currently applied detection methods. Other factors that might have contributed to the failure of detection include the low recovery yield obtained by the applied concentration methods and the small volume of some of the samples collected. Additionally, because of the genetic diversity of norovirus, it could be possible that our RT-PCR assay might be unable to detect some of the strains. To test the possibility that the norovirus-specific primers were inhibited in the RT-PCRs, the extracted RNA from a stool sample, previously known to be positive for norovirus, was mixed with the extracted RNA from a surface water sample. After they have been tested by RT-PCR and southern blotting hybridization, both the mixed RNA and the stool sample extracted RNA tested positive for norovirus RNA, in contrast to the RNA of collected samples that tested negative.

To test the possibility that the negative results obtained for hepatitis A virus and astrovirus were due to some sort of error during the RT-PCR, two different sets of primers, RT-PCR and reagents/kits were used to detect them. However, none of the samples collected tested positive for the genome of these two viruses.

CONCLUSION

This report is describing for the first time the circulation of adenoviruses in sewage and water environmental samples collected from and around Al-Zarqa River, Jordan by a mean of molecular methods. Adenoviruses were detected more

frequently than enteroviruses, and therefore, they can be used as an index for the presence of human pathogenic viruses in water environment.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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