Environmental monitoring of Aichi virus and human bocavirus in samples from wastewater treatment plant, drain, and River Nile in Egypt

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ABSTRACT

Wastewater plays a major role in water pollution causing transmission of several viral pathogens, including Aichi virus (AiV) and human bocavirus (HBoV), associated with gastrointestinal illness in humans. In this study, we investigated the presence of AiV and HBoV in aquatic, sludge, sediment matrices collected from Abu-Rawash wastewater treatment plant (WWTP), El-Rahawy drain, Rosetta branch of the River Nile in Egypt by conventional polymerase chain reaction (PCR). AiV RNA was detected in 16.6% (2/12), 8.3% (1/12), 8.3% (1/12), 22% (16/72), 12.5% (3/24), 4% (1/24), and 0/24 (0%) of untreated raw sewage, treated sewage, sewage sludge, drainage water, drain sediment, river water, and river sediment, respectively. On the other hand, HBoV DNA was detected in 41.6% (5/12), 25% (3/12), 16.6% (2/12), 48.6% (35/72), 29% (7/24), 3/24 (12.5%), 4% (1/24) of untreated raw sewage, treated raw sewage, sewage sludge, drainage water, drain sediment, river water, and river sediment, respectively. This study provides data on the presence of these viruses in various types of water samples that are valuable to environmental risk assessment. In addition, the current study demonstrates the importance of environmental monitoring as an additional tool to investigate the epidemiology of AiV and HBoV circulating in a given community.

Key words | Aichi virus, bocavirus, gastroenteritis, sediment, sewage, water

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INTRODUCTION

Diarrhea is a leading cause of infant and child morbidity and death worldwide (Liu *et al.* 2012). It is caused by consumption of contaminated water or food (e.g., via inadequate sewage and water treatment systems, sanitation facilities, and washing food with contaminated water), person-to-person contact, direct contact with contaminated feces, and poor personal hygiene (UNICEF/WHO 2009; Moors *et al.* 2013). Aichi virus (AiV) and human bocavirus (HBoV) are viral agents that have been documented worldwide in various reports as potential causes of diarrheal disease among children (Yamashita *et al.* 1991; Bergallo *et al.* 2017; Chuchaona *et al.* 2017; Kumar *et al.* 2018; Rikhotso *et al.* 2018).

AiV type 1 (AiV-1) is a member of the family *Picornaviridae* and possesses a positive-sense single-stranded RNA genome (Reuter *et al.* 2011). Based on nucleotide sequences of the conserved 3CD junction region of the viral genome, AiV-1 is divided into three genotypes (A, B, and C) (Yamashita *et al.* 2000; Ambert-Balay *et al.* 2008). The virus has been documented to be the causative agent of diarrhea in humans since it has been detected in 0.9–4.1% of sporadic cases of acute diarrhea in children, with genotypes A and B being dominant (Kaikkonen *et al.* 2010; Jonsson *et al.* 2012; Kumar *et al.* 2018). The virus has been detected in different water matrices such as surface waters in Venezuela, wastewater samples in Tunisia, and in wastewater

doi: 10.2166/wh.2019.075

and river samples in Japan (Alcala *et al.* 2010; Sdiri-loulizi *et al.* 2010; Kitajima *et al.* 2011).

HBoV, belonging to the family *Parvoviridae*, is a negative-sense single-stranded DNA virus (Guido *et al.* 2016). HBoV is genetically divided into four subtypes (HBoV-1 to HBoV-4). HBoV-1 was the first detected from samples of the respiratory tract in 2005 (Allander *et al.* 2005) and it is commonly associated with acute respiratory infections and illness. HBoV-2, 3, and 4 were initially identified in fecal samples from patients with gastroenteritis (Arthur *et al.* 2009; Kapoor *et al.* 2010). Based on data reported by Guido *et al.* (2016) for gastrointestinal infections worldwide, Mexico and Russia have mostly low HBoV prevalence (1.3% and 1.4%, respectively); conversely, the highest prevalence of HBoV has been reported for Bangladesh (63.0%), Tunisia (58.3%), and Nigeria (29.2%). HBoV was identified in 81%, 79.1%, and 51% of sewage samples collected from the United States, Italy, and Norway, respectively (Blinkova *et al.* 2009; Myrmel *et al.* 2015; Iaconelli *et al.* 2016). Also, HBoV was detected in 40.8% and 37.5% of river water samples collected from Egypt and Italy, respectively (Hamza *et al.* 2009; La Rosa *et al.* 2017).

Several enteric viruses (rotavirus, adenovirus, astrovirus, and norovirus) have been studied in the water environment in Egypt (EL-Senousy *et al.* 2014; Elmahdy *et al.* 2019a; Shaheen & Elmahdy 2019). However, the presence of AiV and HBoV in environmental samples is unknown in Egypt. Therefore, the objective of this study was to evaluate the presence of these viruses in sewage, drainage water, and river water from Egypt, as a useful approach to describe the presence of both viruses in the Egyptian environment.

MATERIAL AND METHODS

Area of the study

Abu-Rawash wastewater treatment plant (WWTP) receives about 80% of wastewater produced from Giza governorate, which has a population of 5.3 million. This WWTP treats the raw sewage to primary treated wastewater then discharges it directly into Barakat drain then to other drains until it reaches El-Rahawy drain which disposes its wastewater directly into the Rosetta branch of the River Nile.

Collection and concentration of wastewater samples

Twenty-four sewage (12 untreated raw sewage and 12 treated sewage) and 12 sludge samples were collected monthly, from October 2017 to September 2018, from Abu-Rawash WWTP. Furthermore, 72 drainage water samples from six sites (S1–S6) at El-Rahawy drain and 24 river water samples from two sites (S7 and S8) at the Rosetta branch of the River Nile were collected monthly from April 2017 to March 2018. During the same period, 24 drain sediment samples from two sites (S3 and S4) at El-Rahawy drain and 24 river sediment samples from two sites (S7 and S8) at the Rosetta branch of the River Nile were collected. A description of the sampling sites is presented in Figure 1. The untreated raw sewage (500 mL), treated sewage (1,500 mL), drainage water (1,500 mL), and river water (1,500 mL) samples were concentrated by the adsorption–elution method, using a negatively charged membrane with an acid (H_2SO_4 solution) rinse procedure (Katayama *et al.* 2002). Sewage sludge (100 g) and sediment samples (100 g) were concentrated by a method described previously by the EPA (US EPA 1992) with a minor modification as described by Schlindwein *et al.* (2009) and the PEG 6000 precipitation method for virus concentration was conducted as described by Lewis & Metcalf (1988). Finally, the concentrated samples were stored at −20 °C until used.

Viral genomic extraction

Nucleic acids were extracted from 240 μL of the eluate to obtain a final volume of 60 μL, using the QIAamp Viral RNA and DNA kits (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. To examine polymerase chain reaction (PCR) inhibition, simian rotavirus (SA-11) and human adenovirus type 2 (AdV-2) were used as internal controls, then the nucleic acid yields of the added SA-11 and AdV-2 were evaluated by PCR and no inhibitory effects could be observed (data not shown).

Detection of AiV-1 RNA by semi-nested RT-PCR

The viral RNA was reversed transcribed by using random primer and the presence of AiV-1 was detected by semi-nested PCR as described by Yamashita *et al.* (2000). The first PCR was carried out with primers 6261 (5′-ACACTCCCACCTCCCGCCAGTA-3′) and 6779 (5′-GGAAGAGCTGGGTGTCAAGA-3′) to amplify

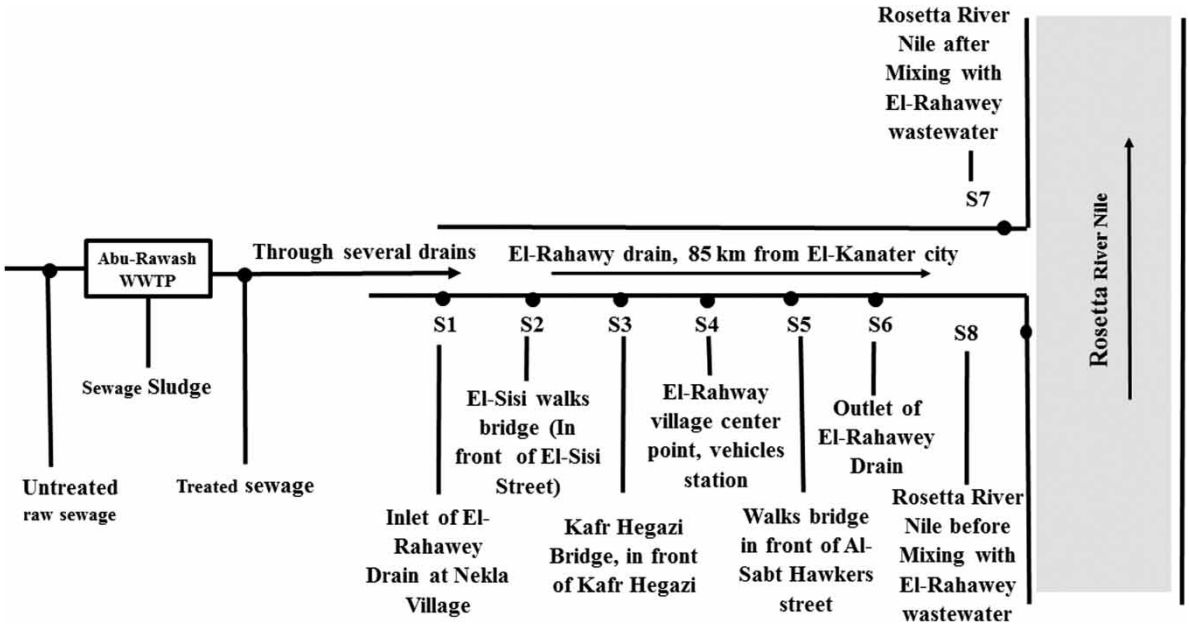

Figure 1 | Description of sampling sites.

a 519 bp sequence of 3CD regions under the following PCR protocol (conducted in Thermo Electron, West Palm Beach, FL, USA): 50 °C for 60 min and 94 °C for 2 min, followed by 40 cycles (each cycle was 94 °C for 30 s, 50 °C for 30 s, and 68 °C for 1 min), then a final extension at 68 °C for 10 min. A semi-nested PCR was performed using the primer pair 6261 and AiMP-R (GCR GAG AAT CCR CTC GTR CC) to amplify a 295 bp segment within the 3CD region under the following PCR program: 95 °C for 15 min and 35 cycles (each cycle was 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s), then a final extension at 72 °C for 10 min.

Detection of HBoV DNA by nested PCR

Nested PCR targeting the VP1/VP2 region to detect also HBoV-2/3/4 species was described previously by La Rosa *et al.* (2016). First round PCR to amplify 543 bp was conducted with primer 234F1 (5′-GAAATGCTTTCTGCT GYTGAAAG-3′) and 234R1 (5′-GTGGATATACCCACA YCAGAA-3′) under thermocycling conditions as follows: 95 °C for 10 min; 40 cycles (94 °C for 30 s, 55 °C for 3 s, and 72 °C for 60 s); with a final step at 72 °C for 10 min. Nested PCR was performed to amplify 382 bp with primer 234F2 (5′-GGTGGGTGCTTCCTGGTTA-3′) and 234R2 (5′-TCTTGRATTTCTTTTCAGACAT-3′) under the same cycling used in the first PCR but with lowering the annealing temperature at 50 °C.

RESULTS

Detection of AiV-1 and HBoV in sewage and sludge samples

As shown in Table 1, AiV-1 was detected in 16.6% (2/12), 8.3% (1/12), and 8.3% (1/12) of the untreated raw sewage, treated sewage, and sewage sludge samples analyzed, respectively. HBoV was detected in five of 12 untreated raw sewage (41.6%), three of 12 treated sewage (25%), and two of 12 sewage sludge (16.6%). Internal controls were used to identify the presence of PCR inhibition and no inhibitory effects were found (data not shown).

Detection of AiV-1 and HBoV in drainage water and drain sediment samples

The presence of AiV-1 and HBoV was analyzed in drainage water and drain sediment (Table 2). AiV-1 was found in 16 out of 72 (22%) drainage water samples and three out of 24 (12.5%) drain sediment samples, whereas HBoV was detected in 35 out of 72 (48.6%) drainage water samples and seven out

Table 1 | Detection of AiV-1 and HBoV in untreated raw sewage, treated sewage, and sewage sludge during October 2017 to September 2018

Sampling date	Untreated raw sewage		Treated sewage		Sewage sludge	
	AiV-1	HBoV	AiV-1	HBoV	AiV-1	HBoV
10/2017	−	+	−	+	−	−
11/2017	−	−	−	−	−	−
12/2017	+	−	−	−	+	−
01/2018	−	+	−	−	−	−
02/2018	−	−	−	+	−	+
03/2018	−	−	+	−	−	−
04/2018	−	−	−	−	−	−
05/2018	−	−	−	−	−	−
06/2018	−	+	−	−	−	−
07/2018	+	+	−	−	−	−
08/2018	−	−	−	+	−	+
09/2018	−	+	−	−	−	−

+, detected; −, not detected.

of 24 (29%) drain sediment samples. The highest detection rates of both viruses were found at S6 and S7 before mixing with the River Nile.

Detection of AiV-1 and HBoV in river water and river sediment samples

As shown in Table 3, only one sample was positive (1/12, 8.3%) for AiV-1 in river water samples collected from S7 whereas AiV-1 was not detected in the river water samples collected from S8 as well as all river sediment samples. On the other hand, HBoV was detected in two out of 12 river water (16.6%) and one out of 12 river sediment (8.3%) samples collected from S7, one out of 12 (8.3%) river water samples and none of the 12 (0%) river sediment samples collected from S8 at the Rosetta branch of the River Nile.

DISCUSSION

The detection and prevalence of AiV-1 and HBoV have been widely reported in many countries worldwide. However, AiV-1 and HBoV prevalence in Egypt remains largely unknown. To our best knowledge, there is only one surveillance study describing detection of HBoV in urban sewage samples

Table 2 | Detection of AiV-1 and HBoV in drainage water and drain sediment during April 2017 to March 2018

Sampling date	S1		S2		S3		(S3)		S4		(S4)		S5		S6	
	AiV-1	HBoV	AiV-1	HBoV	AiV-1	HBoV	AiV-1	HBoV	AiV-1	HBoV	AiV-1	HBoV	AiV-1	HBoV	AiV-1	HBoV
04/2017	−	+	−	−	−	+	+	−	−	+	−	+	+	+	+	−
05/2017	−	−	−	+	−	+	−	+	−	−	−	−	−	+	−	+
06/2017	−	−	−	+	−	+	−	−	+	−	+	−	−	−	−	+
07/2017	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−
08/2017	−	−	+	−	+	−	−	−	−	−	−	−	+	−	+	+
09/2017	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−
10/2017	−	+	−	+	−	+	−	+	−	−	−	−	−	+	−	+
11/2017	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−
12/2017	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−
01/2018	−	−	−	−	−	−	+	+	−	−	−	−	−	−	+	−
02/2018	−	+	−	−	−	+	−	−	−	+	+	−	+	−	−	+
03/2018	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	+

+, detected; −, not detected; the numbers in parentheses refer to the sites of sediment samples.

Table 3 | Detection of AiV-1 and HBoV in river water and river sediment during April 2017 to March 2018

	S7		(S7)		S8		(S8)	
Sampling date	**AiV-1**	**HBoV**	**AiV-1**	**HBoV**	**AiV-1**	**HBoV**	**AiV-1**	**HBoV**
04/2017	−	−	−	−	−	−	−	−
05/2017	−	+	−	−	−	−	−	−
06/2017	−	−	−	−	−	−	−	−
07/2017	−	−	−	−	−	−	−	−
08/2017	+	−	−	+	−	−	−	−
09/2017	−	−	−	−	−	−	−	−
10/2017	−	−	−	−	−	+	−	−
11/2017	−	−	−	−	−	−	−	−
12/2017	−	−	−	−	−	−	−	−
01/2018	−	−	−	−	−	−	−	−
02/2018	−	+	−	−	−	−	−	−
03/2018	−	−	−	−	−	−	−	−

+, detected; −, not detected; the numbers in parentheses refer to the sites of sediment samples.

(Hamza *et al.* 2017). Thus, there is a lack of data concerning the detection and prevalence of AiV-1 and HBoV in clinical samples and its dissemination in the aquatic environment. Therefore, the aim of the current study is to demonstrate the occurrence of AiV-1 and HBoV in environmental samples.

Environmental specimens, particularly urban sludge, contain several organic and inorganic compounds (e.g., polyphenols, humic acids, and heavy metals) that are toxic and might form complexes with the extracted nucleic acids, inhibiting the enzyme amplification (Schlindwein *et al.* 2010). Thus, semi-nested and nested PCR methods were applied for the extracted nucleic acids from the collected samples in order to increase the specificity of detection and eliminate any false-positive results.

AiV-1 RNA has been detected by semi-nested RT-PCR analysis in untreated raw sewage (16.6%), treated sewage (8.3%), and sewage sludge (8.3%) samples. Five previous studies have reported the detection of AiV in environmental water samples and found different prevalence rates. In Venezuela, AiV RNA was detected in five of 10 (50%) sewage samples (Alcala *et al.* 2010), but in Tunisia, only 15 of 250 (6%) tested untreated sewage and treated sewage samples contained RNA of AiV (Sdiri-loulizi *et al.* 2010). In Italy, AiV was detected in six of 48 (12.5%) tested sewage samples (Di Martino *et al.* 2013). Much higher prevalence was reported in studies from Japan and the Netherlands: raw sewage, 91.7% (11/12); treated sewage, 92% (11/12); river water, 60% (36/60), and surface water, 100% (8/8) (Kitajima *et al.* 2011; Lodder *et al.* 2013; Thongprachum *et al.* 2018). This may be because they conducted nested RT-PCR which may have increased the sensitivity of RT-PCR for AiV detection.

On the other hand, the nested PCR analysis identified HBoV in five of 12 untreated raw sewage (41.6%), three of 12 treated sewage (25%), and two of 12 sewage sludge (16.6%) samples. HBoV detection rates in untreated raw sewage and treated sewage samples are significantly lower than those detected in a previous study conducted at two WWTPs in Egypt (Hamza *et al.* 2017). In comparison with results from other countries, HBoV DNA was detected in 69% and 3% of sewage and surface water samples, respectively, in a study from Uruguay. In Norway, HBoV DNA was identified in 51% and 28% of untreated raw sewage and treated sewage, respectively. Much higher occurrence of HBoV was documented in studies from Italy and Germany where this virus was detected in 97.1% and 40.8% of raw sewage and river water samples, respectively (Hamza *et al.* 2009; Iaconelli *et al.* 2016). These differences in the frequencies of AiV and HBoV detection can be explained by the differences in the geographical area, concentration methods, detection methods, and the primers used in the detection.

No seasonal prevalence was observed for both viruses, which is in agreement with previous studies (Bastien *et al.*

2006; Kitajima & Gerba 2015). However, other studies demonstrated that AiV-1 and HBoV infections had a higher incidence rate in summer or in winter (Yip *et al.* 2014; Jiang *et al.* 2016). The true incidence and seasonality of these pathogens thus remain unknown. This study is limited due to the absence of infectivity test for positive viruses; however, the high prevalence of both viruses in untreated raw sewage may indicate a widespread circulation of both viruses among the Egyptian population.

Interestingly, the number of positive samples for both viruses increased gradually from site 1 to site 6 at El-Rahawy drain (Table 2); this observation may be due to the additional agricultural and urban wastewater which discharged directly without treatment along El-Rahway drain. Furthermore, the detection rates for both viruses were also higher in river water and sediment samples collected from the Rosetta River Nile at site 7 after mixing with El-Rahawy wastewater than river water and sediment samples collected from site 8 before mixing with this wastewater (Table 3). Thus, our results revealed a direct link between El-Rahway drain and contamination of this river.

The detection of AiV and HBoV in treated sewage revealed the difficulty in removing these viruses thoroughly by the procedure applied in the current sewage treatment. In fact, it has already been observed in previous reports that processes of sewage treatment, when present, are not completely effective in removing all or some viruses and therefore human viruses are constantly discharged into the environmental waters (Le Guyader & Pommepuy 2002; Espinosa *et al.* 2008). These data highlight the important role of sewage contamination in the dissemination of the viral infection in the community. Indeed, sewage can spread disease and contaminate drinking or irrigation waters, which then lead to diarrhea in children. Thus, the legislative measures for viral surveillance as part of assessing the microbial risks in surface water should seriously be considered to decrease the risks of adverse effects on both human health and the environment (Sdiri-loulizi *et al.* 2010). Detecting the presence of AiV and HBoV in treated water is one of our objectives in the future.

In this study, the detection rates of AiV-1 and HBoV were higher in wastewater and surface water than in their sediments. This was in agreement with a study conducted on detection of rotavirus, adenovirus, and hepatitis A virus in water and sediment samples where the detection rates of these viruses were higher in water than sediment samples (Elmahdy *et al.* 2016). However, it has been reported that sediments can serve as a potential reservoir of human enteric viruses which can be released again into the water environment as a result of sediment agitation by storm action, boating, dredging, etc. (Alm *et al.* 2003; Searcy *et al.* 2006; Salvo & Fabiano 2007). Moreover, our detection rate of HBoV as DNA virus was higher than AiV-1 as RNA virus and this finding is in agreement with previous studies (Espinosa *et al.* 2008; Elmahdy *et al.* 2019b) where DNA viruses are more stable against the environmental conditions than RNA viruses.

This study is the first documentation of AiV circulation in Egypt as well as the first work where the occurrence of HBoV was analyzed in sewage sludge, drainage water, drain sediment, river water, and river sediment in Egypt, highlighting the possible role of AiV-1 and HBoV as emerging viruses implicated in diarrhea diseases among the Egyptian population. The findings of this study are limited due to the absence of clinical data. Thus, we did not compare our environmental data with the clinical data where this study provides the only surveillance results on AiV-1 and HBoV in the aquatic environment. However, one study from Egypt reported the prevalence of HBoV in 2% of diarrhea stool samples (El-Mosallamy *et al.* 2015). Furthermore, data from sewage can provide data on the presence of enteric viruses in a natural pool of thousands of individual samples.

Taken together, AiV and HBoV may naturally occur in water environments and they are likely to occur if inadequately treated sewage is released in surface water, recreational waters, and water for bivalve cultivation and irrigation of crops. Although a one-year environmental monitoring on AiV-1 and HBoV was conducted successfully in this study, a long-term surveillance of both viruses should be performed in the Egyptian environment and population to control AiV-1 and HBoV infections.

CONCLUSION

This study is the first report to evaluate the prevalence of AiV-1 and the second report of the presence of HBoV in sewage in Egypt. Our findings show a wide circulation of AiV-1 and HBoV in sewage and river water. Although there is no evidence of waterborne transmission for AiV-1

and HBoV, the frequent presence of both viruses in sewage and river waters suggests that AiV-1 and HBoV are widely distributed in the Egyptian population. Future research on the presence, quantitative PCR, and sequencing of AiV-1 and HBoV in both clinical and environmental samples, including drinking water, are needed to understand the potential role of wastewater in the transmission of AiV-1 and HBoV to humans.

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First received 5 April 2019; accepted in revised form 11 July 2019. Available online 22 August 2019