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RESEARCH ARTICLE

Quantification of human adenovirus in irrigation water-soil-crop continuum: are consumers of wastewater-irrigated vegetables at risk?

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Abstract

Because of health concerns regarding the presence of enteric viruses in wastewater effluents, this study was designed to investigate the occurrence of human adenovirus (HAdV) in the irrigation water-soil-crop continuum. Viral particles were extracted from wastewater and wastewater- or water-irrigated soil and crop samples and analyzed using real-time PCR. Concentration of fecal indicator bacteria (FIB) were also determined. Quantitative microbial risk assessment was performed to determine the HAdV illness risk associated with the consumption of wastewater-irrigated vegetables**.** HAdV-F was detected in 74% of wastewater effluent samples with a mean concentration of 38 Genomic Copy (GC)/mL. HAdV was also detected in wastewater-irrigated soil (2×10^2 GC/g) and crop (< 10 GC/g) samples, with no statistically significant difference in concentrations between wastewater- and freshwater-irrigated samples. The results showed no correlation between concentrations of FIB and HAdV in the analyzed samples. Mean probability of illness risk from consumption of wastewater-irrigated vegetables was 4×10^{-1} per person per year (pppy) which was about two orders of magnitude higher than the proposed value by WHO (10^{-3} pppy) for safe reuse of wastewater. This finding suggests that the wastewater reuse for irrigation of vegetables eaten raw could pose a threat to human health with respect to the risk of viral illness, signifying stricter management of wastewater reuse. However, because of uncertainties in the QMRA model, particularly the ratio of infectious to non-infectious virus particles, more data is required to validate the predicted risk. This information is especially important in arid and semi-arid regions where high temperatures, UV radiation intensity, and desiccation can efficiently inactivate microorganisms in the environment.

Keywords Agricultural reuse · Gastroenteritis · Illness risk · Human adenovirus · Wastewater

Introduction

Freshwater shortage has become an increasingly serious problem in recent years as a result of climate change, urbanization, and population growth particularly for agricultural purposes (Becerra-Castro et al. 2015). Wastewater reuse for agricultural irrigation is becoming more prevalent around the world because there is an interest in the use of alternative water sources for overcoming water scarcity and consequently achieving food security.

While agricultural wastewater reuse is a priority for arid and semi-arid regions, there are concerns regarding the presence of pathogenic microorganisms in wastewater and their dissemination via wastewater into the environment, which may cause a variety of environmental and health problems (Symonds et al. 2014; Corpuz et al. 2020). Among pathogenic microorganisms, enteric viruses need special attention because they are excreted at high levels from infected individuals (up to 10^{12} viruses per gram feces) (Mok et al. 2014). Furthermore, enteric viruses with a low infectious dose as low as 10 viral particles cause a broad spectrum of diseases (Bitton 2011). Several outbreaks of enteric viruses originating from wastewater have been documented around the world (Bitton 2011; Sano et al. 2016).

Although viruses could be removed in wastewater treatment plants (WWTPs) through sedimentation, predation by other microorganisms, and disinfection, the efficiency of

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these mechanisms is not clear and may vary for different viral types (Symonds et al. 2014). For example, the attachment of viruses to solid particles and subsequent sedimentation in wastewater processes are dependent on both particle surfaces and virus characteristics (Symonds et al. 2014; Corpuz et al. 2020). On the other hand, due to their physicochemical features, enteric viruses are more resistant to disinfection procedures such as chlorination than pathogenic bacteria (Bitton 2011; WHO 2011).

Among enteric viruses, double-stranded DNA viruses are more resistant to disinfectants and severe environmental conditions than single-stranded RNA viruses (Kundu et al. 2013). Human adenoviruses (HAdVs), which are non-enveloped enteric viruses with linear double-stranded DNA, have high survival characteristics in the environment (Hewitt et al. 2013). Double-stranded viruses such as HAdV have the ability to repair damaged DNA during replication in the host cells and, therefore, are generally more resistant to UV radiation than single-stranded ones (Corpuz et al. 2020). HAdV consist of 7 species (HAdV-A through HAdV-G) including over 54 serotypes (Ziros et al. 2015) which cause infections affecting the respiratory tract, the eyes, and the gastrointestinal tract. They might be spread through the respiratory or fecal–oral pathways. Most cases of adenovirus-associated gastroenteritis are related to subgroup F adenoviruses (HAdV-F), which comprises two serotypes, types 40 and 41 which are considered as the second major cause of childhood gastroenteritis after rotavirus (Bitton 2011). HAdV-F as an emerging pathogen with significant persistence in the environment has been considered by the US EPA on the drinking water candidate of contaminant list (EPA 1998; Bitton 2011).

These aspects highlight HAdV-F as a health concern for the safe reuse of treated wastewater in agricultural activities especially for crops eaten raw (Sano et al. 2016). In other words, the presence of HAdV-F in wastewater effluent represents a potential threat to public health through agricultural reuse of wastewater. Therefore, in arid and semi-arid regions in which the agricultural reuse of wastewater may play a fundamental role in the security of water and food, it is necessary to identify the presence of human enteric adenoviruses in wastewater and health risks associated with the agricultural reuse of wastewater. Although some studies have reported the presence of HAdVs in wastewater (Hewitt et al. 2013; Sidhu et al. 2018; Elmahdy et al. 2019; Gonzales-Gustavson et al. 2019; Nour et al. 2021), few studies are available that have investigated human health risks associated with the presence of HAdVs in aquatic environments (Kundu et al. 2013; Gonzales-Gustavson et al. 2019; Purnell et al. 2020). Furthermore, fate of HAdVs in the irrigation water-soil-crop continuum, especially in arid and semi-arid regions where exposure to sunlight plays an important role in viral decay (McMinn et al. 2020), is currently unknown. The climate conditions of arid and semi-arid areas, including high ambient temperature, ultraviolet (UV) radiation intensity, and low humidity, are important factors that could impact the survival rate of microorganisms in the environment (McMinn et al. 2020; Shamsizadeh et al. 2021).

Therefore, the present study was designed to quantify HAdV-F concentration in the irrigation water-soil-crop continuum under different irrigation water qualities in a semi-arid region. Furthermore, based on the measured viral concentrations in wastewater-irrigated vegetables, the potential health risk of HAdV-F (illness endpoint) associated with consumption of wastewater-irrigated vegetables was estimated using the quantitative microbial risk assessment (QMRA) approach.

Materials and methods

Study site and sample collection

For estimation of HAdV concentrations in wastewater, effluent samples from two large WWTPs in Isfahan were analyzed biweekly during a one-year period from February 2019 to January 2020. Isfahan is located in the central part of Iran with a semi-arid climate with an average annual temperature of 16.7 °C and an average annual rainfall of 130 mm. Both WWTPs use the activated sludge process as secondary treatment, and treated effluent from WWTP A enters into a local river while effluent of WWTP B is released into agricultural fields. Wastewater samples were collected between the hours of 09:00 am and 12:00 pm in 500-mL sterilized glasses.

To investigate the fate of HAdV-F in the irrigation water-soil-crop continuum, irrigation wastewater/water along with irrigated soil and vegetable samples were collected at harvesting events in experimental and farm fields. Lettuce and spring onion were collected in an experimental field in the WWTP A, which was irrigated with treated wastewater (TWW) and fresh water (tap water) as control as described previously (Farhadkhani et al., 2018). Other vegetables, including leek, purslane, and spring onion, were collected from commercial agricultural fields located in the peri-urban area of Isfahan that were irrigated with different water qualities, including TWW, a channel which is mainly fed by TWW, and fresh water (ground water). Each field was divided into three equal sections and three soil or vegetable samples were collected per each section and mixed as a composite sample. Soil samples were obtained from the 0–20 cm topsoil layer. Crops were harvested 2–3 days after the last irrigation event and prepared for microbial analysis as described by Farhadkhani et al. (2020). Forty-two soil and crop samples in total were analyzed as presented in Table 1.

Table 1 Number of analyzed samples based on irrigation water type

Sample type	Number of samples	Water irrigation	Wastewater irrigation
Wastewater effluent	65	-	-
WWTP[a] A	38	-	-
WWTP B	27	-	-
Water	9	-	-
Soil	21	9	12
Crop	21	9	12

[a]Wastewater treatment plant

All samples were collected in sterilized glasses or bags and immediately transported on ice to the laboratory for further analysis. All samples were analyzed for the presence of fecal indicator bacteria (FBI) and HAdV-F.

Detection of fecal indicator bacteria

For detection of coliforms and *Escherichia. coli* as indicators of fecal pollution, the enzymatic assay by LMX broth was used, and the results were expressed as MPN/100 mL or MPN/g (Farhadkhani et al. 2020).

Detection of HAdV-F

Sample preparation

For detection of HAdV-F, 200 mL of wastewater and water samples were concentrated by the adsorption-elution method as described in *standard methods* (APHA 2012; Moazeni et al. 2017).

For extraction of viruses from soil and crop samples, polyethylene glycol 8000 precipitation method was used as described in ISO/TS 15,216–2:2019(E) (Anonymous 2019). Briefly, edible parts of crop samples were washed in tap water and then coarsely chopped. To a soil and crop volume yielding 5 and 25 g, respectively, 40 mL of Tris/glycine/beef extract (TGBE) was added. The mixture (pH = 9) was shaken at room temperature on a horizontal shaker (60 oscillations/min) for 20 min, then particles were separated by a fine mesh strainer and supernatant was centrifuged at 6000 × g for 30 min at 4 °C. To the supernatant (adjusted to pH 7), polyethylene glycol 8000 and NaCl (to give final concentrations of 100 g/L PEG and 0.3 mol/L NaCl) were added. The mixture was incubated at 5 ± 3 °C with constant rocking at around 60 oscillations/min for 60 min and then centrifuged for 30 min at 6000 × g and 4 °C. The pellet was resuspended in 500 µL of PBS and chloroform/butanol mixture for soil and vegetable samples, respectively. The chloroform tube was centrifuged and the aqueous phase was transferred to a fresh tube and retained for DNA extraction at − 20 °C.

DNA extraction and quantification of HAdV-F

Detection of HAdV-F in concentrates was performed by real-time PCR analysis. Viral nucleic acid was extracted and purified from the concentrates by using the High Pure Viral Nucleic Acid kit (Roche, Molecular Biochemicals Ltd, Mannheim, Germany) as per manufacturer's instructions and eluted in 35 µL elution buffer.

Real-time PCR was performed using specific primers (AD40/41F 5′-CAGCCTGGGGAACAAGTTCA-3′ (Rajal et al. 2007), and HEX245R 5′-ACTTTGTAAGARTARGCG GTKTC-3′ (Simmons and Xagoraraki 2011)) in an ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The PCR analysis was performed in a final volume of 15 µL contained 7.5 µL 2 X reaction mix (Ampliqon, Denmark), 0.2 µM of each primer, 5 µM $MgCl_2$, 0.25 mg/mL BSA (Sigma-Aldrich), and 2 µL template DNA. The cycling parameters were 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 59 °C for 45 s. Finally, a melting curve analysis was performed to eliminate the possibility of false-positive results.

For quantification of HAdV-F, a standard curve was generated using tenfold serial dilutions of a quantified plasmid (10^5 to 10^0 copies per reaction). The limit of detection (LOD).

was obtained as 1 genomic copy per reaction. Positive (plasmid) and negative controls were included in all reactions. For confirmation of the real-time PCR results, a few samples tested positive for HAdV-F were randomly selected, and the PCR products were subjected to sequencing and BLAST analysis.

The efficiency of DNA extraction was determined with a comparison of the average copy number of AdV type 5 in spiked samples before and after extraction in an identical manner as the field samples except by using specific primers for total adenovirus (F: 5′-GCCACGGTGGGGTTTCTA AACTT-3′ and R: 5′-GCCCCAGTGGTCTTACATGCACAT -3′ (Heim et al. 2003)).

Statistical analysis

SPSS 26.0 software was used for statistical analysis. The maximum, minimum, mean, and standard deviation of HAdV, FIB, and physicochemical parameters in the two WWTPs were determined using descriptive analysis. The concentrations of HAdV and FIB in wastewater samples were not normally distributed (as determined by a Kolmogorov–Smirnov test). Therefore, the non-parametric Mann–Whitney *U* test was used to compare HAdV and FIB concentrations between the two WWTPs. Spearman's correlation was implemented to determine the potential relationship between the analyzed parameters. Significance was defined as a *P* value of ≤ 0.05.

Risk analysis

The possible health risks associated with the consumption of wastewater-irrigated vegetables were assessed using a QMRA framework. The QMRA employs a quantitative method based on simulation techniques and dose–response modeling and includes four steps: hazard identification, exposure assessment, dose–response evaluation, and risk characterization (Haas et al. 2014).

Hazard identification

HAdV-F with high persistence in the environment is considered as a viral marker. On the other hand, HAdV-F is the causative agent of gastroenteritis which may be transmitted through wastewater-irrigated crops. Therefore, in the study, the risk of HAdV-F associated with the consumption of wastewater-irrigated vegetables was estimated.

Exposure assessment and dose–response

Consumption of wastewater-irrigated vegetables may contribute to illness risk of HAdV. In the study for the first time, measured values of HAdV-F in wastewater-irrigated vegetables instead of concentrations in wastewater were used in QMRA model to estimate the risk of viral infection associated with the consumption of wastewater-irrigated vegetables. However, QMRA was also used to estimate the illness risk of HAdV based on the concentration of viral particles in effluent samples as described previously (Moazeni et al. 2017) (Supplementary file). The numbers of infectious adenovirus ingested by a person were calculated through the following equation:

$$N_{veg} = C_{veg} \times R^{-1} \times I_{\mathrm{fr}} \times M \quad (1)$$

Where N_{veg} (median tissue culture infectious dose ($TCID_{50}$)/person.day) is the number of infectious HAdV-F ingested by a person through consumption of wastewater-irrigated vegetables; C_{veg} is the number of HAdV-F (genomic copy number/g) in wastewater-irrigated vegetables as determined by the real-time PCR; R is the recovery efficiency of the detection method; I_{fr} is the fraction of infectious HAdV-F (1000 genomic copy number = 1 $TCID_{50}$) (Mcbride et al. 2013); and M (g/person. day) is the vegetable daily consumption for adults (≥ 18 years) which was obtained from the most recent nationwide survey (CASPIAN-IV).

The probability of infection risk (P_i(d), per person per day) from consumption of wastewater-irrigated vegetables was estimated by Beta-Poisson model based on the probability distribution for the calculated ingestion dose (veg) (Teunis et al. 2016; Gonzales-Gustavson et al. 2019). The dose–response equation is presented as below:

$$P_i(d) = 1 - (1 + \frac{N_{veg}}{\beta})^{-a} \quad (2)$$

Where Nveg is the ingested dose of HAdV-F and the α and β are parameters of the Beta-Poisson model (Teunis et al. 2016).

Using infection as the endpoint may overestimate the estimated risk because all infections don't cause illness. Therefore, it has been proposed that illness is considered as the endpoint in risk analysis (Kundu et al. 2013). The probability of illness after infection (P_{ill}; per person per day) was calculated as follows:

$$P_{\mathrm{ill}}(d) = P_i(d) \times I_{\mathrm{ill/inf}} \quad (3)$$

where $I_{\mathrm{ill/inf}}$ is the proportion of adenovirus-infected persons who become ill, which was considered as 0.5 based on the epidemiological studies in developing countries (Kundu et al. 2013).

The annual risk of HAdV-F illness (P_{ill}(A)) is estimated by Eq. (4):

$$P_{\mathrm{ill}}(A) = 1 - [1 - P_{\mathrm{ill}}(d)]^{\mathrm{n}} \quad (4)$$

Where n is the number of days in a year that an individual is exposed to the HAdV-F through consumption of wastewater-irrigated vegetables. The number of exposure days was considered from 180 to 240 days based on the harvesting period of vegetables in the study region. Input parameters used in the risk analysis of HAdV-F are presented in Table 2.

Risk characterization

The numbers of infectious HAdV-F ingested by a person and the probability of illness risk from exposure to HAdV-F through wastewater-irrigated vegetables consumption were calculated using Monte Carlo simulations with 10,000 iterations. In order to minimize the uncertainty in estimated risk, whenever possible, input data was presented as probability distribution rather than point estimate. Sensitivity analysis was performed to determine the influence of variation in the input parameters on the estimated infection risk using Spearman's rank-order correlation. All modeling and analysis were implemented using R version 3.3.1.

The parameters with the highest relative effects are considered to be the most sensitive input parameters. The acceptable annual illness risk level proposed by the WHO (10^{-3} per person per year, pppy) for safe reuse of wastewater, was used for interpreting the magnitude of the risk assessment outcomes (Mara et al. 2007).

Table 2 QMRA model input parameters

Parameter	Symbol	Unit	Distribution type (values)	References
HAdV-F concentration in wastewater-irrigated vegetables	Cveg	GC[a] mL^{-1}	Gamma (0.465, 4.651)	This study
Recovery efficiency of virus	R	%	Uniform (45, 55)	This study
Fraction of infectious particles to genome copies	I_{fr}	Fraction	Constant (1×1000^{-1})	(Mcbride et al., 2013)
Vegetable consumption (M)	M	g day^{-1}	Normal (11.81, 14.20)	Questionnaire, this study
Number of exposure days in a year	n	Days	Uniform (180, 240)	This study
Parameter for the Beta-Poisson model	α	-	Lognormal (1.631, 1.044)	(Teunis et al., 2016)[b]
	β	-	Lognormal (1.029, 0.824)	(Teunis et al., 2016)[b]

[a]Genomic Copy; [b]Distribution in this study

Results and discussion

Because conventional WWTPs are not very efficient in removing enteric viruses such as HAdV, agricultural reuse of wastewater effluent may pose a threat to public health. Our results showed a high frequency of detection (74%) of HAdV-F in effluent of WWTPs. HAdV-F was detected in 79% and 66% of effluent samples of WWTP A and WWTP B, respectively. Adenoviruses were detected in 100% of wastewater and combined sewer overflow (CSO) discharge samples in the USA (Fong et al. 2010). In a study, 141 sewage samples from 22 WWTPs in 10 Italian regions, collected in 2013, showed the presence of HAdV in 85 out of 141 (60%) raw wastewater samples and HAdV type 41 was the most frequently detected HAdV (45 samples) (Iaconelli et al. 2017). Pina et al. (1998) also reported a high frequency of detection of AdVs in sewage in Spain, higher than other enteric viruses. In contrast, in treated effluent from two WWTPs in Morocco, HAdV was detected only in %22 (2⁄9) of samples. Detected viruses were identified as HAdV-B and HAdV-D, and HAdV-F was not detected in any of samples (Amdiouni et al. 2012). Elmahdy et al. (2019) investigation in an urban WWTP in Egypt showed presence of HAdV in 27 (84.4%) of raw sewage and 16 (50%) of secondary treated sewage samples in a range of about 104–106 GC/mL. In an investigation in Riyadh, Saudi Arabia, HAdV was detected in 44% and 61% of samples of two WWTPs (Nour et al. 2021).

As shown in Table 3, high concentrations of FIB and HAdV-F were detected in the effluent samples. HAdV was detected with a mean of 38 genomic copies (GC) per milliliter in effluents of WWTPs with the higher mean concentration in WWTP A (48 GC/mL). While in a previous study, higher efficiency of WWTP A in the removal of suspended solids and biochemical oxygen demand was reported (Aali et al. 2014); no significant difference in HAdV concentrations was observed between the two WWTPs. In agreement with our results, AdVs were detected at a geometric mean value of 7 PCR units/mL in effluents of six WWTPs located in different prefectures in Japan and were the most abundant among the tested viruses (Katayama et al. 2008). Gonzales-Gustavson et al. (2019) reported a mean concentration of 2.06×10^4 and 9.62×10^3 GC/100 mL for adenovirus in effluent samples of two WWTPs located in the northeast of

Table 3 Microbial and physicochemical characteristics of wastewater effluent samples

Parameter	WWTP A			WWTP B			Sig[a]
	Min	Max	Mean ± standard deviation	Min	Max	Mean ± standard deviation	WWTPA/WWTP[b]
Sample temperature (°C)	14	30	22 ± 4.4	16	29	22 ± 4.15	ns
pH	7	8	-	7	8	-	ns
Total coliforms (TC) (MPN 100 mL^{-1})	3.3×10^1	2.4×10^7	$2.6\times10^6\pm5.3\times10^6$	3.9×10^2	1.1×10^9	$6\times10^7\pm2.2\times10^8$	*
Fecal coliforms (FC) (MPN 100 mL^{-1})	ND[c]	1.1×10^7	$1.2\times10^6\pm2.5\times10^6$	2×10^3	2.4×10^7	$6.7\times10^6\pm5.2\times10^6$	*
E. coli (MPN 100 mL^{-1})	ND	1.1×10^7	$6.5\times10^5\pm2.4\times10^6$	3.6×10	1.5×10^7	$5.5\times10^6\pm8.2\times10^5$	*
HAdV-F (genomic copy mL^{-1})	ND	5.7×10^2	$48\pm1.08\times10^2$	ND	3.6×10^2	$22\pm7.2\times10$	ns

Sig Significance, *Test was significant at $P\leq0.05$. *ns* not significant.

[b]Comparison of the parameters of WWTP A with the parameters of WWT B.

[c]Not detected

Spain. Hewitt et al. (2011) also reported AdVs, in a range of 0.7–3.26 $\log_{10}$ infectious unit per liter (IU/L) and 2.97–6.95 $\log_{10}$ GC/L by cell culture assay and qPCR, respectively, from ten New Zealand WWTPs. Culturable AdVs were detected in 19 of 30 (63%) samples with the highest concentration of 3.26 $\log_{10}$ IU/L in the effluent samples. Furthermore, AdV was more commonly detected than enterovirus (EV) and norovirus (NoV) (Hewitt et al. 2011). However, lower concentrations of adenovirus (8.02×10^2 GC/L) were reported in the effluent of three WWTPs in sub-tropical Brisbane located in Southeast Queensland, Australia (Sidhu et al. 2018). A relatively high concentration of HAdV has also been reported in other aquatic environments. In study of Kundo et al. (2013), adenovirus type 40/41 was detected in 11% of 73 surface water samples, ranging from 147 to 4117 genomes per liter. In an urban catchment area in Singapore, mean and median concentrations for HAdV were 1.2×10^3 GC/L and 9.0×10^2 GC/L, respectively (Vergara et al. 2016).

Although FIB were also detected with high concentrations in effluent samples (Table 3), no correlation was found between these bacteria and HAdV. This finding suggests no usefulness of FIB to predict AdVs presence in effluent. The lack of relationship between viruses and FIB has also been reported in other studies (Petrinca et al. 2009; Donia et al. 2010; Rames et al. 2016; Moazeni et al. 2017; McMinn et al. 2020).

HAdV-F was detected in all the seasons with the highest frequency as well as concentration in the autumn (Fig. 1). In study of Elmahdy et al. (2019),HAdV was detected throughout the year with a peak in winter at 100% (16/16), followed by autumn at 68.7% (11/16). However, Katayama et al. (2008) reported that the concentrations of EVs and AdVs were mostly constant all year round in Japan.

Our results showed detection of HAdV-F in wastewater-irrigated soil and crop samples. However, the virus was also detected in soil and crop samples irrigated with freshwater, and there was no significant difference between the content of HAdV-F in water- and wastewater- irrigated fields (Table 4). This is not surprising, the presence of HAdV could be the result of land application of biosolids as a common amendment in farm fields of the study region and suggests that caution must be exercised (Amoah et al. 2007). However, the difference between concentration of *E. coli* in wastewater-irrigated and water-irrigated soil may in part be related to the lower persistence of coliform bacteria than adenoviruses in the environment (Bitton 2011). In arid and semi-arid regions, the decay of microorganisms in soil and on crop surface may happen quickly (Farhadkhani et al. 2018). Enteric viruses with their non-enveloped structure show resistance to environmental degradation and desiccation (Mok et al. 2014). In contrast, a relatively low concentration of HAdV (8.97 GC/g), but a relatively high concentration of *E. coli* (2.59×10^2 MPN/g) was found on wastewater-irrigated vegetables with no significant difference between the two types of irrigation. Persistence of pathogens on plant surfaces is highly affected by intensity of sunlight and temperature (Sampson et al. 2017; McMinn et al. 2020), resulting in low concentrations of HAdV on crop surfaces in a semi-arid region. In other words, survival of microorganisms on plant surfaces is generally short if they are exposed to sunlight, high temperatures, and drying (Masclaux et al. 2014; Sales-Ortells et al. 2015) Furthermore, it has been reported that most of the human viruses present in the environment are non-infectious (Kundu et al. 2013). However, as indicated in other studies, detection of high concentrations of *E. coli* on wastewater- as well as water-irrigated crops could be related to the external source of fecal pollution such as birds and/or wild and domestic animals, which are commonly found in agricultural fields (Benami et al. 2013; Farhadkhani et al. 2018). Although the results showed the contamination of vegetables with FIB and HAdV (Table 4), it seems that there is a considerable reduction of the microorganisms in soil and crop samples irrigated with wastewater. This was expected, considering the high temperature, low humidity, and UV intensity in the semi-arid regions could effectively inactivate microorganisms (McMinn et al. 2020) However, because of low infectious dose of enteric viruses, the presence of HAdV-F in low numbers on vegetables eaten raw may be a concern from public health point of view. This finding highlights the importance of information regarding the infectivity of detected HAdV in the environment, especially in arid and semi-arid regions where weather conditions may significantly influence viral viability/infectivity (Bitton 2011; McMinn et al. 2020).

Risk analysis showed a high risk of illness from consumption of wastewater-irrigated vegetables. The mean annual illness risk was 4×10^{-1} (95% CI: 2.6×10^{-1}–4.9×10^{-1}) per person per year (pppy) which was higher than the WHO

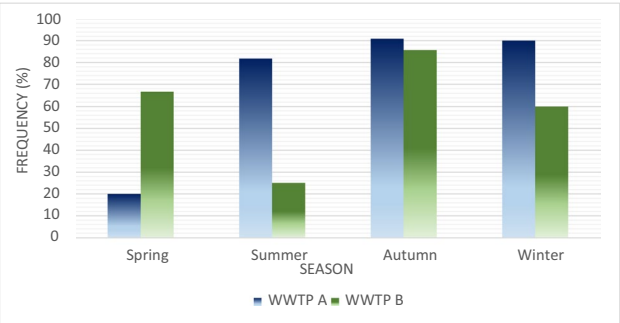

Fig. 1 Frequency of HAdV-F detection in WWTPs effluent in different seasons

Table 4 Microbial characteristics of water and wastewater- irrigated samples

Sample type	Microorganism	Irrigation type		Sig[b]
		Water irrigation[a]	Wastewater irrigation[a]	
Irrigation water	*E.coli* (MPN 100 mL^{-1})	9×10^{-1}	2.48×10^{4}	*
	HAdV-F (GC[d] mL^{-1})	ND	9.63×10^{1}	*
Soil	*E.coli* (MPN g^{-1})	8.12×10^{1}	2.71×10^{2}	*
	HAdV-F (GC g^{-1})	9.39×10^{1}	2.03×10^{2}	ns[c]
Crop	*E.coli* (MPN g^{-1})	1.18×10^{1}	2.59×10^{2}	ns
	HAdV-F (GC g^{-1})	6.37	8.97	ns

[a]Mean concentration; [b]Mann-Whitney was significant at $P<0.05$; [c]not significant; [d]genomic copy

health target of $<10^{-3}$ pppy recommended for safe reuse of wastewater (Fig. 2).

A QMRA study based on the numbers of HAdV in tertiary treated wastewater samples of two WWTPs was conducted in Spain, in which the estimated annual illness risk was about one order of magnitude higher than the benchmark value proposed by WHO. The estimated annual risk was reported 3×10^{-2} pppy for tertiary treated wastewater with a mean concentration of 6.7 GC/mL of adenovirus (Gonzales-Gustavson et al. 2019). Considering the lower concentration of HAdV in tertiary treated effluent than which detected in our wastewater effluents (about one $\log_{10}$), the reported adenovirus risk by Gonzales-Gustavson et al. (2019) is consistent with our results. The results highlight further removal/inactivation of viruses by WWTPs or by in-field measurements. Generally, QMRA studies on various enteric viruses have shown a relatively high infection risk or disease burden associated with the consumption of wastewater-irrigated crops, especially for produce eaten raw (Mok and Hamilton 2014; Sales-Ortells et al. 2015). Mok et al. (2014) reported a disease burden of 4.7×10^{-4} to 4.4×10^{-3} disability adjusted life years (DALY) pppy from the consumption of lettuce irrigated with wastewater treated by stabilization ponds. High norovirus infection risk was reported from the consumption of storm water-irrigated lettuce (Lim et al. 2015). However, the estimated mean illness in children for adenovirus type 40/41 via the ingestion of recreational waters was 3.5%, which is below the level (3.6%) considered tolerable for recreational activity (Kundu et al. 2013).

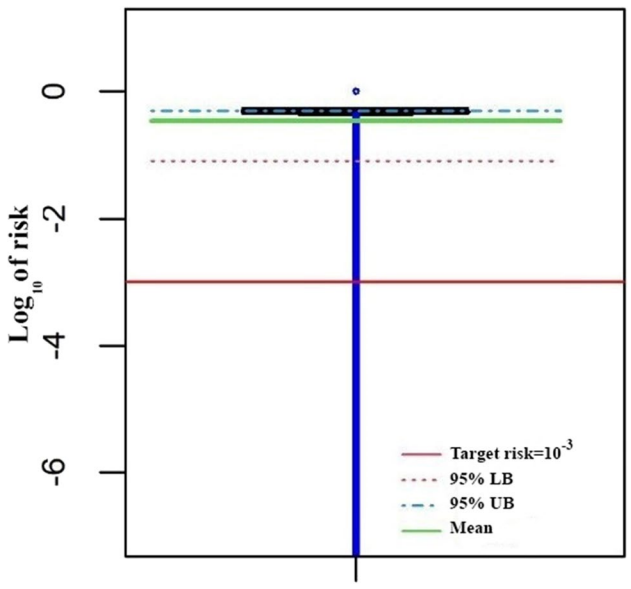

Fig. 2 The box plot of estimated annual HAdV-F illness risk (pppy) in consumers of wastewater-irrigated vegetables in comparison to the reference level of 10^{-3} pppy (red line)

On the other hand, enteric viruses present higher risks than bacterial and protozoan pathogens (Mara 2008). Higher illness risk was reported from HAdV and NoV than from the bacterial pathogens, *Campylobacter* and *Salmonella* (Chhipi-Shrestha et al. 2017). However, the estimated risk from HAdV-F may be over- or under-estimated because of the considered ratio of 0.5 for illness/infection. It is possible that viral infections occur asymptomatic in a large portion of population, especially in developing countries. The exact factors contributing to disease are not well known, but age and immune status of the infected individuals play an important role in the symptomatic infection or illness (Gentile and Micozzi 2016). Uncertainty in the dose–response model is another important factor which probably under- or over-estimated the infection risk (Mok et al. 2014).

Results of sensitivity analysis revealed that the levels of infectious HAdV-F in wastewater-irrigated vegetables were the most influential factor on the estimated risk (Fig. 3). In other words, variation in the ratio of viral genome to infectious viral particles and consequently the ingested HAdV through consumption of wastewater-irrigated vegetables can have a significant bearing on the predicted risk, as seen in Fig. 3 and has been reported by others (Kundu et al. 2013; Sales-Ortells et al. 2015; Gonzales-Gustavson et al. 2019). We used a ratio of 10^3 GC to $TCID_{50}$ for estimation of the infectious HAdV in crop samples, whereas Kundu et al. (2013) considered a range from 10^3 to 10^5 with a most likely value of 10^4. Since the GC to $TCID_{50}$ ratio depends on the virus inactivation in the environment and could be

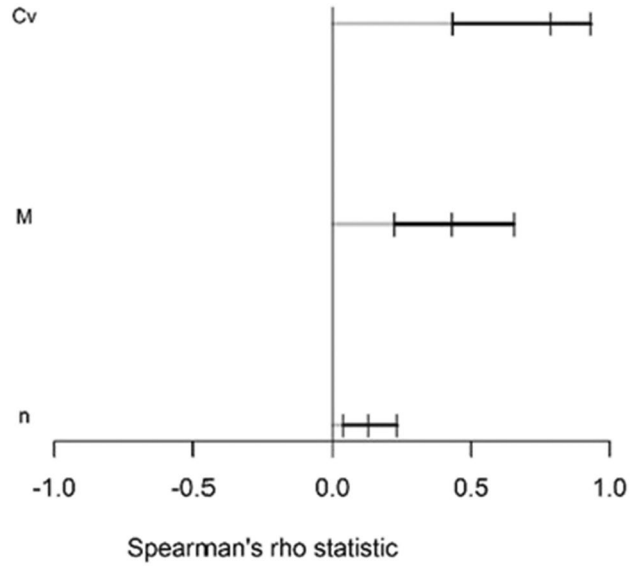

Fig. 3 The Spearman's rank correlation between the input variables and the HAdV-F illness risk

different for each region based on the climate conditions, caution should be taken in using PCR-based data in the risk assessment (Lodder et al. 2015). It has been reported that viral-genome based measurements may not be suitable in health risk assessment due to the lack of sufficient data about the relationship between genomic copy and infectious viral particle numbers in various environments (Kundu et al. 2013). However, efforts have been made to relate PCR measurements with health effects, such as enterococcus qPCR guidelines for beaches which are recommended by US EPA (Vergara et al. 2016). Temperature, exposure of the viruses to direct sunlight, weather humidity, and virus type are important factors that influence the survival/infectivity of viruses in the environment and on crop samples (Lodder et al. 2015; Courault et al. 2017; McMinn et al. 2020). In other words, persistence of pathogens on crop surfaces is generally short if they are exposed to sunlight and drying (Masclaux et al. 2014; McMinn et al. 2020). The results of a study on MS2 virus on lettuce surface as a surrogate for enteric viruses showed about 3 logs inactivation of the virus after 25 h at 30 °C and exposure to artificial sunlight (Carratalà et al. 2013). Therefore, due to the fact that ratio of infectious to non-infectious virus particles may significantly decrease over time especially in arid and semi-arid regions with high temperature and UV index, detection of genomic part of the viruses could lead to the overestimation of risk (Lodder et al. 2015). Further research, therefore, on viral decay factors is needed for increasing the accuracy of risk estimations (Mok and Hamilton 2014). Vergara et al. (2016) also reported that the absence of infectivity data for both NoV and HAdV creates uncertainty for the estimation of risks associated with the recreational surface waters (Vergara et al. 2016). It is important to note that the longer interval time between the last irrigation and harvesting of vegetables is an important influential parameter which reduces the risk of contamination of crops by microorganisms (Li and Wen 2016; Shock et al. 2016; Farhadkhani et al. 2018) and consequently the infection risk. Therefore, the treated wastewater may be safely reused for irrigation of crops with little need for water and the ability to withstand a longer interval time between the last irrigation and harvesting.

Variation in the daily consumption of vegetables had also a significant effect on the estimated annual infection risk. A sensitivity analysis on infection risk from NoV revealed that the model parameters with higher influence on the probability of disease were the concentration of NoV in the effluent and the consumption of lettuce (Mok et al., 2014; Sales-Ortells et al., 2015). Gonzales-Gustavson et al. (2019) also reported that the concentrations of HAdV in wastewater and ingestion rate of lettuce are the most important parameters affecting the output of risk analysis.

Conclusions

This work aimed to study the risk of HAdV-F associated with consumption of wastewater-irrigated vegetables based on the QMRA model. The results of present study indicate high concentrations of HAdV-F in the effluent of WWTPs. However, the results of field study showed that irrigation water quality alone may not be a source of adenovirus in soil and on crops. The results of risk analysis revealed that the annual HAdV illness risk was about 2 orders of magnitude higher than proposed by WHO, for consumers of wastewater-irrigated vegetables. These findings highlight the need for more attention to the health implications of viruses associated with the agricultural reuse of wastewater. Since the virus level in vegetable samples was the major factor influencing the estimated risks, more inactivation of viruses in the environment through a longer interval time between the last irrigation and harvesting could be used as a risk mitigation technique in the agricultural reuse of wastewater. However, as the extent of the risk is highly dependent on viability/infectivity of HAdV, additional researches on the viral decay rate, especially in semi-arid regions and consequently the proportion of infectious viruses to the detected viral particles would be useful for increasing the accuracy of risk estimations.

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Declarations

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