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ORIGINAL PAPER

Detection of Norovirus and Hepatitis A Virus in Strawberry and Green Leafy Vegetables by Using RT-qPCR in Egypt

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Abstract

There is an upward trend of consumption of organic fresh vegetables due to consumer demand for healthy foods without chemical additives. On the other hand, the number of food borne outbreaks associated with contaminated fresh produce has raised, being human norovirus genogroup I (GI), GII and hepatitis A virus (HAV) the most commonly reported causative agents. This study aimed to detect the presence of these viruses in green leafy vegetables (watercress, leek, coriander, and parsley) and strawberry using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Samples were collected from the Egyptian regions of Kalubia, Giza, and Mansoura. Overall HAV average occurrence in fresh strawberry was 48% with a mean concentration of 6.1×10^3 GC/g; Also NoV GI overall average occurrence was 25% with a mean concentration of 9.7×10^2 genome copies (GC)/g, while NoV GII was 40% with a mean concentration of 2.4×10^3 GC/g. For strawberry collected directly from Kalubia farms, neither HAV nor HNoV GI & GII were detected. In green leafy vegetable samples, the occurrence of HAV was 31.2% with a mean concentration of 9.2×10^4 GC/g, while occurrence of NoV GI and NoV GII were 20% and 30% with a mean concentrations of 1.1×10^4 and 2.03×10^3 GC/g, respectively. In conclusion, the importance of a virus surveillance program for soft fruits and fresh vegetables is highlighted by the outcomes of this study. Our findings should help with the management and control of microbial concerns in fresh foods, reducing the danger of consuming contaminated foods.

Keywords Green leafy vegetables · Strawberry · Hepatitis A virus · Noroviruses · Real-time PCR

Introduction

Foodborne viral infections are considered one of the most important public health threats all over the world that can pose a significant burden to the economies of the developed and developing countries, and It is likely to have serious adverse to food safety consequences. Unlike bacteria, viruses don't grow or multiply in foods but foods may become contaminated with human enteric viruses and transmit infections. Among the major foodborne enteric viruses that can be responsible for gastroenteritis or other clinical manifestations include hepatitis A virus (HAV) and human noroviruses (HNoV), which are also the most frequently detected in the aquatic environment beside the human adenoviruses, rotavirus species A, hepatitis E virus, human astroviruses, and enteroviruses (Chen et al., 2007; Elmahdy et al., 2016, 2019, 2020; Fongaro et al., 2013; Mangeri et al., 2020; Pavoni et al., 2021; Ruscher et al., 2020). HAV and noroviruses are currently recognized as the most causative agents related to foodborne diseases (FAO/WHO, 2008a, 2008b; Newell et al., 2010; Terio et al., 2020; Dirks et al., 2021), which are included among the 31 foodborne pathogens in US (Scallan et al., 2011). HNoV and HAV are non-enveloped enteric viruses, positive sense, and single-stranded RNA viruses of approximately 7.5 kilobases each and thus they are more stable in the environment than enveloped viruses (Alidjinou et al., 2019; Elmahdy et al., 2018). They are primarily transmitted via the fecal–oral

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route, either by person-to-person contact or ingestion of contaminated food or water; they may be also shed in vomitus (Matthews et al., 2012; Kotwal & Cannon, 2014; Marti et al., 2016). Many developed countries, such as the United States and the European Union, have been forced to impose strict food safety regulations based on existing surveillance systems for viral contaminants in foods as a result of increasing number of foodborne outbreaks (Aboubakr & Goyal, 2019; Grace, 2015). Fresh fruits such as strawberry can be contaminated via the people handling, processing food and contaminated irrigated water source than environmental contamination (Koopmans & Duizer, 2004; Morin & Picoche, 2008; FAO/WHO, 2008a, 2008b; Jeong et al., 2013). Direct consumption of fresh produce (uncooked food) without proper washing is risky as they are considered the most important vehicle for the foodborne transmission (Bassett & McClure, 2008; Li et al., 2015). Due to some challenges with cell culture, the recently published ISO standard technique for viral detection in food is based on the quantitative RT-PCR method rather than the cell culture method. (ISO 15216-1:2017, 2017). Globally, the highest annual cases related to foodborne viral illness caused by norovirus were (124,803,946) infections with 34,929 deaths, indicating that it has a mortality rate of 0.028. HAV comes in the second place which causes 13,709,836 cases and 27,731 deaths with 0.202 of fatality rate (WHO, 2015; Lee & Yoon, 2021). The main objective of the present study was to evaluate and investigate the presence of HAV, HNoV GI and GII in naturally contaminated fresh vegetables. The samples of coriander, parsley, watercress, leek and strawberries were selected for this study to recommend safe practices for the production and consumption of these soft fruits and leafy greens vegetables.

Materials and Methods

Study Area

Three governorates in Egypt were selected for sample collection in the current study two of these governorates (Giza, Dakahlia) are represented the big strawberry retail markets for human consumption while the third governorate is Kalubia which contains the strawberry farms: (1) Giza governorate which is the third-largest city in Egypt and situated on the west bank of the Nile River, (2) Mansoura city, It is the capital of the Dakahlia Governorate, on the east bank of the Damietta branch of the Nile River, in the Delta region, and (3) Kalubia Governorate Located in Lower Egypt. It is situated north of Cairo in the Nile Delta region as shown in Fig. 1.

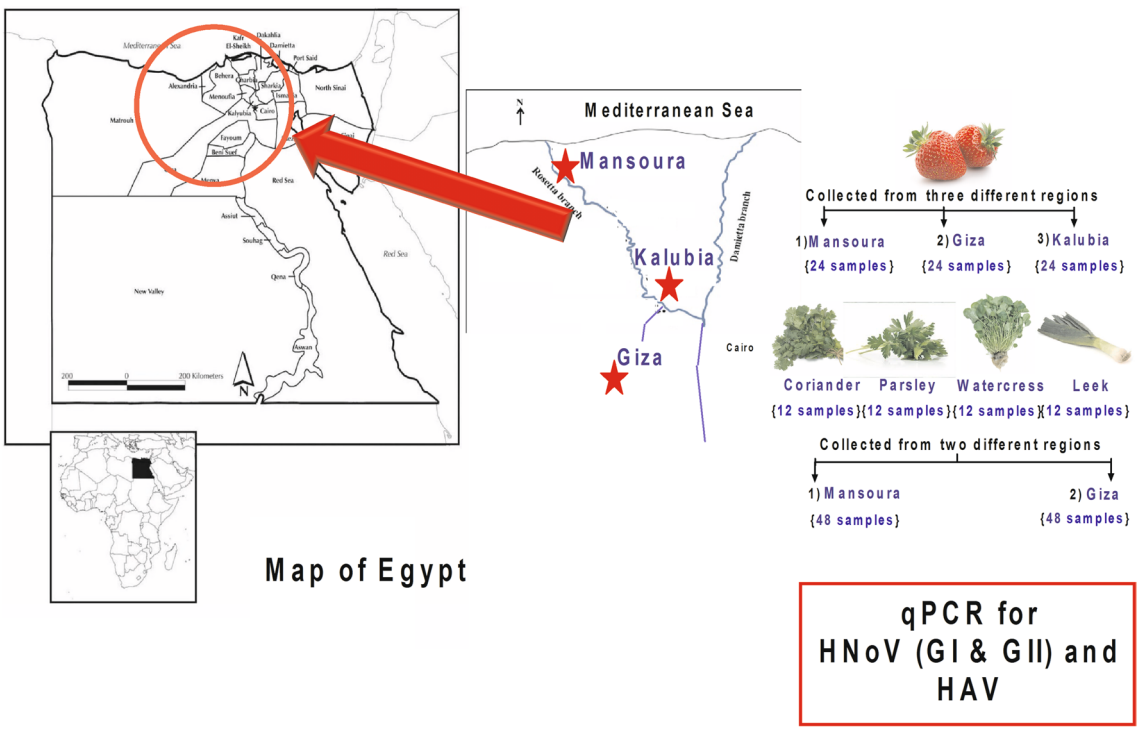

Fig. 1 Field of strawberry and green leafy vegetables samples collection at different sites in Mansoura, Giza and Kalubia

Sample Collection

Strawberry

Forty-eight strawberry samples unpackaged were collected from six sites from two regions in Egypt (Giza and Mansoura, 24 samples from each region) to represent the end of the strawberry production chain handler for human consumption (i.e., retail establishments such as the popular vegetable markets). In addition, twenty-four strawberry samples were collected directly from two strawberry farms in Kalubia (12 strawberry samples from each farm), to represent the beginning of the strawberry production chain (i.e., directly from the field) over a period of 4 months (November & December 2019 and January & February 2020) according to the harvest season as shown in Fig. 1.

Green Leafy Vegetables

A total of 96 green leafy vegetables (Coriander, Parsley, watercress and Leek) were collected from cultivated land near the riverbed from two regions (Giza and Mansoura) directly from the farms. From each region, 48 green leafy vegetables (12 from each type) were collected over a period of 4 months (November & December 2019 and January & February 2020) as shown in Fig. 1.

Effect of Freezing on HNoV and HAV

All strawberry samples collected during this study were primarily divided into two subsamples (25 g each) when arrived at lab: (1) the first divided subsample was going directly for the viral genome concentration for HNoV and HAV detection, (2) The second divided subsample was freeze preserved at –20 °C for 1 week until further detection to show the effect of freezing on the virus genome.

Viral Concentration

The method defined in ISO 15216-1:2017 was used to detect HNoV and HAV concentrations in green leafy vegetables and strawberries samples (ISO 15216-1:2017, 2017). Briefly, in a sterile plastic bag containing 50 ml of Tris–glycine buffer (TGBE: 100 mM Tris–HCl, 50 mM glycine, and 1% beef extract, pH 9.5; in case of strawberry, 30 units of pectinase from *Aspergillus niger* (Sigma-Aldrich, Germany) was added to TGBE to prevent jelly formation), 25 g of each green leafy vegetable (in small parts) or strawberry samples was transferred in a sterile plastic bag, separately. After 20 min of continuous rocking (approximately 70 oscillations/min) at room temperature to detach the virus from the surface of the samples, the sample was divided and transferred into clean centrifuge tubes. The vegetable or strawberry matter was discarded after centrifugation at 10,000×*g* for 30 min at 4 °C, and the eluates were transferred into clean tubes and changed to neutral pH (7. with 1.0 N HCl. With gentle rocking at 4 °C for 60 min, the pH adjusted eluates were combined with 0.25 volume of 50 percent (w/v) polyethylene glycol 8000/1.5 M NaCl. After centrifugation at 10,000×*g* for 30 min at 4 °C, the pellets were then dissolved in 500 µL of 10 mM Phosphate buffer solution (PBS) and stored at – 20 °C until use.

Viral RNA Extraction and Virus Detection by Real-Time qPCR

The final processed samples were applied to the extraction of viral RNA nucleic acid using a QIAamp MiniElute Virus Spin Kit (Qiagen, Germany) as instructed by the manufacturer. To examine PCR inhibition and monitor the efficiency of extraction and viral recovery, a representative sample was taken from each type of green leafy vegetables samples and also strawberry samples collected during this study and inoculated with 4.7×10^8 GC/ml of (MNV-1) as sample process control virus (SPCV) and as an external amplification control (EAC) as described in ISO 15216-1. EAC added to an aliquot of RNA sample. The degree of RT-PCR inhibition in each tested sample is obtained by comparing these results with the results of EAC RNA in the absence of sample RNA to reveal the false negative results and no inhibitory effects could be observed. The amplification efficiency (E) of the quantitative real-time RT-PCR was calculated using the slopes (S) of the regression lines. For molecular detection of (HAV), the qPCR was performed in accordance with Jothikumar et al. (2005), HNoV GI and GII were performed in accordance with Kageyama et al. (2003) and MNV-1 were performed in accordance with Lee et al. (2005), using one-step Rotor-Gene (RG) Probe RT-PCR Kit with thermal cycler profile as described in Table 1. Real-time PCR mixture (25 µL) contained 5 µL RNA extract, 12.5 µL 2 × RG PCR MM and RG RT-PCR MM reagents (Qiagen, Germany), 0.8 µM of each primer, 0.2 µM of TaqMan probe for each corresponding virus type, and nuclease-free water up to 25 µL. PBS was used as the negative nucleic acid extraction control. This qPCR mixture was transferred into the Rotor-Gene Q system. At the end of each stage of the annealing, fluorescence data are measured by the provided Rotor-Gene software. A serial tenfold dilution of nucleic acids was used to dilute the inhibitor and increase PCR efficiency. All amplifications were performed in duplicate. For each experiment, a positive control PCR amplicon was created by cloning the amplicon into a plasmid (pGEM-T Easy Vector (Promega) for HAV strain HM175; and pCR2.1-TOPO vector for HNoV GI &GII), and amounts of purified plasmid DNA were determined using a Nano Drop spectrophotometer. Standard curves were created by running tenfold

Table 1 Primers, probes, and thermal cycling conditions used in this study to amplify HAV, HNoV GI, HNoV G II, and MNV-1

Virus	Primer and probe sequence 5′–3′	Thermal cycling conditions	Source of references
HAV	F: GGTAGGCTACGGGTGAAAC R: GCGGATATTGGTGAGTTGTT Probe: FAM-CTTAGGCTAATACTTCTATGA AGAGATGC –TAMRA	48 °C for 30 min, followed by 95 °C for 15 min, then 40 cycles of 95 °C for 10 s, 55 °C for 20 s and 72 °C for 15 s	Jothikumar et al. (2005)
HNoV GI	F: CGYTGGATGCGNTTYCATGA R: CTTAGACGCCATCATCATTYAC Probe: FAM -AGATYGCGATCYCCTGTCCA-TAMRA	50 °C for 2 min, followed by 95 °C for 10 min, then 45 cycles of 95 °C for 15 s and 56 °C for 1 min	Kageyama et al. (2003)
HNoV GII	F: CARGARBCNATGTTYAGRTGGATGAG R: TCGACGCCATCTTCATTCACA Probe: FAM-TGGGAGGGCGATCGCAATCT-TAMRA	50 °C for 2 min, followed by 95 °C for 10 min, then 45 cycles of 95 °C for 15 s and 56 °C for 1 min	Kageyama et al. (2003)
MNV-1	F: ACGCTCAGCAGTCTTTGTGA R: CTGGCCTCAGAGCCATTG Probe: FAM-CGCTGCGCCATCACTCATCC-TAMRA	95 °C for 30 s. then 40 cycles of at 95 °C for 15 s and 60 °C for 45 s	Lee et al. (2015)

serial dilutions of plasmids. Each test used ultra-pure water as a non-template control to ensure that the assay was free of contamination. Standard HAV and HNoV curves were prepared with a tenfold serial dilution of the DNA standard ranging from 5×10^{1} to 5×10^{9} GC/reaction. Non-template controls (NTC) consisting of DEPC water were included in each assay. Table 1 lists all of the primers and probes used in this analysis, as well as the virus's thermal cycling conditions.

To investigate virus recovery in all virus detection assays, we used murine norovirus (MNV) as a process control virus in this study. A representative strawberry and vegetable samples (previously tested negative for MNV by RT-qPCR) were inoculated with murine norovirus 1 (MNV-1) suspension (4.7×10^{8} GC/mL) as a viral process control. Briefly, 25 g from each vegetable and strawberry sample was weighed separately and cut into small square pieces in case of vegetable samples. After, 100 µL of MNV-1 was distributed as drops on the surface of each sample. Then, all the samples were in biosafety cabinet and allowed to dry for 30 min followed by viral concentration process. The recovery percentages were obtained according to the following equation Recovery $(\%) = GC_{\text{obtained titer}} / GC_{\text{Spiked titer}} \times 100\%$. Triplicates of these samples were made. Each experiment included non-contaminated strawberry samples that served as negative controls.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism version 5.0 (USA) technology. The critical *P*-value for the test was set at 0.05. The Pearson correlation was used to test the associations between viral distributions in different samples. A one-way variance analysis was used to compare the mean viral loads of the samples. To find discrepancies, student t-tests and ANOVA tests are used.

Results

Recovery rate and Limit of MNV-1 Detection in Spiked Strawberry and Green Leafy Vegetables Samples

The qPCR limit of detection was determined using the maximum dilution at which virus quantification was possible (The detection limits for HAV were 2×10^{1} GC/g in 25 g of samples and whereas the detection limit for HNoV GI, HNoV GII, and MNV-1 were 3×10^{1} GC/g). In the spiked experiment a representative strawberry and vegetable samples (coriander, Parsley, watercress, and Leek); the MNV-1 recovery was almost 2.7×10^{8} GC/g (57.4%), for strawberry and green leafy vegetables samples.

Detection of HAV and HNoV in Fresh Strawberry Samples

Table 2 shows the presence of HAV and HNoV GI and GII in fresh strawberry samples. HAV was detected in 46% (11/24) and 50% (12/24) of Giza and Mansoura collected samples, respectively. While HNoV GI was found in 21% (5/24) and 29% (7/24) of the samples collected in Giza and Mansoura, respectively, as shown in Table 3. Occurrence of HNoV GII was most common than HNoV GI with a percentage of 38% (9/24) and 42% (10/24) in the samples collected in Giza and Mansoura, respectively, as shown in Table 4.

Table 2 Detection of HAV in fresh and frozen strawberry samples

Virus	Time of sample	Sampling sites					
		Fresh strawberry			Frozen strawberry		
		End of strawberry chain supply		Beginning of strawberry chain	End of strawberry chain supply		Beginning of strawberry chain
		Giza (6 sites retails) (GC/g)	Mansoura (6 sites retails) (GC/g)	Kalubia (2 farms)	Giza (6 sites retails) (GC/g)	Mansoura (6 sites retails) (GC/g)	Kalubia (2 farms)
	Nov. 2019	3.6×10^3	2.6×10	–	3.1×10^3	2.4×10^3	–
		–	–	–	–	–	–
		4.3×10^3	–	–	4.2×10^3	–	–
		–	–	–	–	–	–
		–	5.3×10^3	–	–	4.1×10^3	–
		5.1×10^3	–	–	3.7×10^3	–	–
HAV	Dec. 2019	3.1×10^3	2.1×10^3	–	2.2×10^3	1.7×10^3	–
		–	–	–	–	–	–
		–	3.1×10^3	–	–	1.1×10^3	–
		–	–	–	–	–	–
		4.2×10^3	–	–	3.6×10^3	–	–
		–	2.4×10^3	–	–	1.9×10^3	–
	Jan 2020	–	–	–	–	–	–
		5.3×10^3	4.1×10^3	–	4.9×10^2	3.5×10^3	–
		–	–	–	–	–	–
		3.6×10^3	3.7×10^3	–	3.2×10^2	4.3×10^3	–
		4.1×10^3	–	–	3.6×10^3	–	–
		–	4.3×10^3	–	–	3.6×10^3	–
	Feb. 2020	–	3.1×10^4	–	–	3.2×10^4	–
		3.1×10^2	–	–	2.9×10^2	–	–
		2.5×10^3	–	–	2.2×10^3	–	–
		–	2.3×10^3	–	–	2.1×10^3	–
		4.5×10^2	3.3×10^3	–	4.1×10^2	2.9×10^3	–
		–	4.3×10^4	–	–	4.3×10^4	–
Number of + ve samples		46% (11/24)	50% (12/24)	0% (0/24)	46% (11/24)	50% (12/24)	0% (0/24)
Average		3.3×10^3	8.9×10^3		2.2×10^3	8.4×10^3	

None of the tested viruses could be detected by RT-qPCR in all samples from Kalubia, which is the direct original source of strawberry in their farms. On the other hand, the strawberry subsamples that were maintained in freezer -20 also yielded the same results as shown in Tables 2, 3 and 4.

Detection of HAV and HNoV in Green Leafy Vegetables Samples

Table 5 shows the results of viruses analysis performed in fresh produce samples. Overall occurrence of HAV was 31.2% with a mean concentration of 9.2×10^4 GC/g. HAV was present in 42% (5/12) of watercress, 33% (4/12) of leek, 33% (4/12) of coriander, 17% (2/12) of parsley, in Giza. In Mansoura, HAV was present in 58% (7/12) of watercress, 33% (4/12) of leek, 17% (2/12) of coriander, 33% (4/12) of parsley. HAV genome copy number in these samples ranged from 2.1×10^3 to 7.1×10^4 GC/g.

The occurrence of NoV GI was 20% with a mean concentration of 1.1×10^4 GC/g. HNoV GI was present in 17% (2/12) of watercress, 25% (3/12) of leek, 17% (2/12) of coriander, 25% (3/12) of parsley in Giza, while in Mansoura, HNoV G I was present in 17% (2/12) of watercress, 25% (3/12) of Leek, 17% (2/12) of Coriander, 17% (2/12) of Parsley. HNoV GI genome copy numbers in these samples ranged from 2.3×10^2 to 4.3×10^3 GC/g. While occurrence of NoV GII was 30% with a mean concentrations of 2.03×10^3 GC/g. HNoV GII was present in 33% (4/12) of watercress, 33% (4/12) of leek, 33% (4/12) of coriander, 17% (2/12) of parsley in Giza. While in Mansoura, HNoV

Table 3 Detection of HNoV GI in fresh and frozen strawberry samples

Virus	Time of sample	Sampling sites					
		Fresh strawberry			Frozen strawberry		
		End of strawberry chain supply		Beginning of strawberry chain	End of strawberry chain supply		Beginning of strawberry chain
		Giza (6 sites retails) (GC/g)	Mansoura (6 sites retails) (GC/g)	Kalubia (2 farms)	Giza (6 sites retails) (GC/g)	Mansoura (6 sites retails) (GC/g)	Kalubia (2 farms)
	Nov. 2019	–	–	–	–	–	–
		–	–	–	–	–	–
		–	1.5×10^2	–	–	1.3×10^2	–
		4.4×10^2	–	–	3.7×10^2	–	–
		–	1.9×10^2	–	–	1.4×10^2	–
		–	-	–	–		–
HNoV GI	Dec. 2019	–	2.3×10^2	–	–	1.4×10^2	–
		–	–	–	–	–	–
		–	–	–	–	–	–
		–	–	–	–	–	–
		–	1.4×10^2	–	–	1.2×10^2	–
		2.1×10^2	–	–	2.0×10^2	–	–
	Jan 2020	–	–	–	–	–	–
		–	2.5×10^2	–	–	2.7×10^2	–
		–	–	–	–	–	–
		–	5.1×10^2	–	–	4.2×10^2	–
		–	–	–	–	–	–
		2.2×10^3	–	–	2×10^3	–	–
	Feb. 2020	3.4×10^3	–	–	3.1×10^3	–	–
		–	–	–	–	–	–
		–	–	–	–	–	–
		–	1.6×10^2	–	–	1.2×10^2	–
		2.2×10^3	–	–	1.5×10^3	–	–
		–	–	–	–	–	–
Number of + ve samples		21% (5/24)	29% (7/24)	0% (0/24)	21% (5/24)	29% (7/24)	0% (0/24)
Average		1.7×10^3	2.3×10^2		1.4×10^3	1.9×10^2	

GII was present in 42% (5/12) of watercress, 33% (4/12) of leek, 33% (4/12) of coriander, 17% (2/12) of parsley. HNoV GII genome copy numbers in these samples ranged from 1.4×10^2 to 4.3×10^3 GC/g (Table 5).

Discussion

Foodborne viruses pose a significant threat to global health, despite the fact these viruses have yet to be taken into account in food safety legislation. The globalization of the food supply has resulted in a rise in the circulation of fresh produce from unknown sources of unknown quality, which may lead to many outbreaks worldwide. The results outcome from this study and also other studies (Cheong et al., 2009; Shaheen et al., 2019; Victor et al., 2021) suggest possible contamination of green leafy vegetables and strawberries from external sources and may be this contamination originated from polluted water or handler of field-workers (Koopmans & Duizer, 2004; Cheong et al., 2009; Hall et al., 2012; Marti & Barrardi, 2016).

Norovirus (NoV) and HAV are two foodborne viruses that have been connected to several foodborne illness outbreaks linked to fresh and frozen berries around the world (Bernard et al., 2012; Severi et al., 2015; Palumbo et al., 2016; Ruscher et al., 2020). In the present study, we investigated four types of green leafy vegetables from two different regions, also we evaluate the presence of these enteric viruses in strawberries collected directly from the farm region (Kalubia) where it's considered as a start point for

Table 4 Detection of HNoV GII in fresh and frozen strawberry samples

Virus	Time of sample	Sampling sites					
		Fresh strawberry			Frozen strawberry		
		End of strawberry chain supply		Beginning of strawberry chain	End of strawberry chain supply		Beginning of strawberry chain
		Giza (6 sites retails) (GC/g)	Mansoura (6 sites retails) (GC/g)	Kalubia (2 farms)	Giza (6 sites retails) (GC/g)	Mansoura (6 sites retails) (GC/g)	Kalubia (2 farms)
	Nov. 2019	–	–		–		–
		–	–	–	–	–	–
		2.1×10^3	–	–	1.8×10^3	–	–
		–	–	–	–	–	–
		–	3.1×10^3	–	–	2.7×10^3	–
		–	2.6×10^3	–	–	2.1×10^3	–
HNoV G II	Dec. 2019	2.9×10^3	–	–	2.6×10^3	–	–
		–	–	–	–	–	–
		–	–	–	–	–	–
		–	–	–	–	–	–
		1.4×10^3	3.3×10^3	–	1.0×10^3	3.1×10^3	–
		–	5.2×10^3	–	–	3.2×10^3	–
	Jan 2020	–	–	–	–	–	–
		–	–	–	–	–	–
		–	3.9×10^2	–	–	3.4×10^2	
		4.8×10^3	2.1×10^2	–	4.5×10^3	1.5×10^2	
		3.4×10^3	1.3×10^3	–	2.9×10^3	1.1×10^3	
		2.6×10^3	–	–	2.1×10^3	–	
	Feb. 2020	1.2×10^2	–	–	1.2×10^2	–	–
		–	2.7×10^3	–	–	2.4×10^3	–
		2.2×10^3	3.1×10^3	–	2.0×10^3	2.1×10^3	–
		–	–	–	–	–	–
		–	1.1×10^3	–	–	0.9×10^3	–
		3.2×10^3		–	2.3×10^2	–	–
Number of + ve samples		38% (9/24)	42% (10/24)	0% (0/24)	38% (9/24)	42% (10/24)	0% (0/24)
Average		2.5×10^3	2.3×10^3		1.9×10^3	1.8×10^3	

strawberry chain production from different region at Egypt with the same time strawberries samples were collected from two retail regions (Mansoura and Giza). Multiple controls, such as viral process control and EAC, must be utilized to confirm the absence of substances that leading to inhibit PCR amplification**.** According to ISO 15216, inhibition rates for RNA isolated from food samples must be less than 75%, and viral process control extraction yields must be better than 1%.In this work, the mean percentages of real-time quantitative inhibition RT-PCR were never more than 75%, and MNV-1 recovery was greater than 1% in 91% of the RNA extracts analyzed. According to the recommendations in ISO 15216, extraction/recovery efficiency must be determined in all tested samples, while some limitation in the current study regarding to representative samples from each type of tested food were tested for extraction and recovery efficiency. In the current study, NoV GI was detected in 25% (12/48) of the collected strawberry samples. Brassard et al. (2012) detected NoV GI in 27% of the strawberry samples that were collected in field-grown strawberries. Recently, contaminated strawberries have also been reported as a source of norovirus outbreaks in the United States (Hall et al., 2012). In Malaysia and Canada, NoV GI was detected in 13.33%, 3.33%, and 25% of green onions, red onions, and strawberries, respectively; with viral concentration between 3.0×10^3 and 5.0×10^1 particles/gram in strawberries samples (Brassard et al., 2012; Hidayah et al., 2011). NoV GII was detected in 39.5% (19/48) of strawberry samples collected during this study from 2 regions (Mansoura and Giza). The occurrence of HAV and HNoV GI and II in

Table 5 Detection of HAV, HNoV GI, and HNoV GII in green leafy vegetables samples

Virus	Time of sample		Sampling sites							
			Giza				Mansoura			
			Watercress (GC/g)	Leek (GC/g)	Coriander (GC/g)	Parsley (GC/g)	Watercress (GC/g)	Leek (GC/g)	Coriander (GC/g)	Parsley (GC/g)
	Nov. 2019	Site 1	–	–	–	–	2.1×10^4	–	–	–
		Site 2	–	–	–	–	2.5×10^4	2.1×10^3	–	
		Site 3	–	–	–	–	–	–	–	1.1×10^4
HAV	Dec. 2019	Site 1	2.1×10^4	4.6×10^3	5.1×10^3	2.1×10^3	3.4×10^3	1.5×10^3	1.1×10^3	2.5×10^4
		Site 2	–	–	–	3.3×10^3	–	–	–	–
		Site 3	–	–	–	–	–	–	–	–
	Jan 2020	Site 1	7.1×10^4	1.9×10^4	3.9×10^4	–	2.2×10^3	3.8×10^4	–	–
		Site 2	–	–	2.6×10^4	–	3.3×10^3	–	–	–
		Site 3	4.3×10^4	–	–	–	–	–	–	3.1×10^3
	Feb. 2020	Site 1	4.1×10^3	$3.5\times10^{4-}$	–	–	4.3×10^3	–	–	–
		Site 2	–		–	–	2.5×10^3	3.1×10^4	–	2.2×10^3
		Site 3	3.2×10^4	2.1×10^3	2.4×10^3	–	–	–	2.5×10^3	–
Number of + ve samples			42% (5/12)	33% (4/12)	33% (4/12)	17% (2/12)	58% (7/12)	33% (4/12)	17% (2/12)	33% (4/12)
Average			3.4×10^4	1.5×10^4	1.8×10^4	2.7×10^3	8.8×10^3	1.8×10^3	1.8×10^3	1×10^4
HNoV G I	Nov. 2019	Site 1	–	–	–	3.5×10^3	–	–	2.1×10^3	–
		Site 2	–	–	–	–	–	2.2×10^3	–	–
		Site 3	–	2.3×10^2	–	4.2×10^3	1.3×10^3	–	–	–
	Dec. 2019	Site 1	–	–	–	–	3.2×10^3	–	–	–
		Site 2	–	3.1×10^2	–	–	–	–	–	2.8×10^2
		Site 3	–	–	–	–	–	4.3×10^3	–	–
	Jan 2020	Site 1	3.2×10^3	2.5×10^2	3.5×10^2	2.9×10^2	–	–	–	6.1×10^2
		Site 2	4.3×10^3	–	–	–	–	–	1.3×10^3	–
		Site 3	–	–	–	–	–	–		–
	Feb. 2020	Site 1	–	–	3.1×10^2	–	–	3.4×10^3	–	–
		Site 2	–	–	–	–	–	–	–	–
		Site 3	–	–	–	–	–	–	–	–
Number of + ve samples			17% (2/12)	25% (3/12)	17% (2/12)	25% (3/12)	17% (2/12)	25% (3/12)	17% (2/12)	17% (2/12)
Average			2.7×10^4	9.6×10^3	1.3×10^4	2.7×10^3	7.5×10^3	1.3×10^4	1.8×10^3	7.5×10^3
HNoV GII	Nov. 2019	Site 1	–	–	–	–	1.7×10^3	1.1×10^3	–	–
		Site 2	–	–	3.3×10^3	–	–	2.3×10^3	–	3.7×10^2
		Site 3	–	–	–	–	–	–	–	–
	Dec. 2019	Site 1	2.5×10^2	–	3.9×10^3	–	–	2.1×10^2	3×10^3	–
		Site 2	4.7×10^2	–	2.7×10^3	–	2.5×10^3	–	2.1×10^3	–
		Site 3	–	2.4×10^3	–	2.1×10^3	–	–	–	–

Table 5 (continued)

Virus	Time of sample	Sampling sites									
		Giza					Mansoura				
		Watercress (GC/g)	Leek (GC/g)	Coriander (GC/g)	Parsley (GC/g)		Watercress (GC/g)	Leek (GC/g)	Coriander (GC/g)	Parsley (GC/g)	
	Jan 2020	Site 1 –	3.6×10^3	–	–		1.9×10^2	–	4.2×10^3	–	
		Site 2 –	3.3×10^3	–	–		–	3.1×10^2	2.5×10^3	–	
		Site 3 –			–		–	–	–	–	
	Feb. 2020	Site 1 3.1×10^3	4.1×10^3	–	–		2.2×10^2	–	–	1.4×10^2	
		Site 2 4.3×10^3	–	–	2.3×10^3		2.5×10^2	–	–	–	
		Site 3 –	–	3.8×10^3			–	–	–	–	
Number of + ve samples		33% (4/12)	33% (4/12)	33% (4/12)	17% (2/12)		42% (5/12)	33% (4/12)	33% (4/12)	17% (2/12)	
Average		2×10^3	3.4×10^3	3.4×10^3	2.2×10^3		9.7×10^2	9.8×10^2	3×10^3	2.6×10^2	

all strawberry tested samples was similar in both fresh and frozen samples indicating that the Freezing for one week is not sufficient to inactivate or degrade RNA viral pathogens and may be longer periods has the ability to degrade virus genome (Koopmans & Duizer, 2004). Furthermore, the current study is also constrained by the lack of an infectivity test for positive samples by qPCR; however, the significant abundance of these viruses in collected samples could imply that they are widely circulated. Finally, testing for viruses in commodities is difficult, and there is considerable debate over interpretation of findings from molecular assays, because these do not provide information on the viability of the pathogens detected (Baert et al., 2011). Although, other reports mentioned that frozen berries are also important source of NoV transmission (Dietrich et al., 2013; Maunula et al., 2009; Sarvikivi et al., 2012). HAV was detected in 48% (23/48) of strawberry collected during the study from 2 regions (Mansoura and Giza). HAV detected in Mansoura 50% (12/24) more than in Giza 46% (11–24). In recent years, there have been numerous reports of hepatitis A outbreaks connected to frozen berries (Fitzgerald et al., 2014; Guzman-Herrador et al., 2014; Ruscher et al., 2020; Wenzel et al., 2014). The findings of the investigations described here, and the detection of HAV in imported frozen strawberries several weeks later, triggered an amendment of import regulations in the European Union (Marti et al., 2021) and African Countries (Faour-Klingbeil & Todd, 2020).

Fresh produce has been identified as an important vehicle for the foodborne transmission (Bassett & McClure, 2008; FAO/WHO, 2008a, 2008b). We also collected a total of 96 green leafy vegetables (Coriander, Parsley, watercress and Leek) from two regions (Giza and Mansoura). HAV was detected in 64.5% (31/48) of the total (coriander, parsley, watercress, and leek) from Giza and Mansoura. The higher concentration of HAV was detected in watercress 50% (12/24) in comparison to the other vegetables, also HAV was detected to higher levels in Mansoura (33.3%) 16/48 than Giza (31.2%) 15/48. Khan et al. (2014) detected HAV in all cultivated vegetables due to exposure to faecalis contaminated irrigation water. Regarding to organic food, a recent study done in Barcelona, Spain on organic lettuce, parsley, and strawberry and the enteric viruses were found in 33.3%, 37.7%, and 16.6%, respectively (Itarte et al., 2021). Furthermore, several HAV outbreaks associated with green leafy vegetables (as green onion) consumption at several restaurants were documented in 2003 at Monaca and Pennsylvania; in 2000 at Kentucky and Florida; in 2003 at Tennessee, Georgia, and North Carolina; and in 1999 at Ohio (Amon et al., 2005; Datta et al., 2001; Dentinger et al., 2001; Wheeler et al., 2005). Interestingly, Bidawid et al. (2000) reported that 9.2% of contaminated lettuce by infectious virus particles comes from contaminated hands. NoV GI was detected in 41.7% of total leafy green vegetable (coriander, parsley, watercress, and leek) from Giza and

Mansoura. NoV GII was detected in 60% (29/48) of total leafy green vegetable (coriander, parsley, watercress, and leek) from Giza and Mansoura. These findings were consistent with other worldwide studies, such as Terio and co-workers found that NoV GII was the most common enteric virus 74.8% of Ready-To-Eat Salads samples (Terio et al., 2020). Watercress was the most contaminated vegetable with NoV GII by (37.5%). Raw vegetable crops (such as leafy greens) and fruits were responsible for 30% and 21%, respectively, of NoV foodborne outbreaks in the U.S. 2009 to 2012 (Hall et al., 2014). Concerning fresh produce outbreaks, NoV was identified as the top cause of outbreaks (40%), according to a comprehensive survey of outbreaks with identified food sources in the U.S. (since 1990 to 2005) (Dewaal & Bhuiya, 2009). Recently, NoV GII was the most common pathogen found (89.50%) of all samples collected from different food categories throughout a 6-year survey in Italy (Pavoni et al., 2021). In recent Egyptian study, Shaheen et al. (2019) detected NoV GI in 34.4%, 40.6%, 31%, and 28% in watercress, green onion, lettuce, and leek samples, respectively, while the virus was detected in (31.2%) of surface irrigation water. A series of outbreaks of NoV GI and NoV GII gastroenteritis related to consumption of lettuce between 18 and 20 January 2010 was documented to Danish authorities (Ethelberg et al., 2010). In Canada and Malaysia, NoV GI was found in 13.33%, 3.33%, and 25% of green onions, red onions, and strawberries, respectively (Brassard et al., 2012; Hidayah et al., 2011). Different prevalence rates of human NoV were found in leafy greens from several countries; 2.9% in Italy (Purpari et al., 2019), 12.4–50% in France (Baert et al., 2011; Loutreul et al., 2014), 5.3% in United Kingdom (Cook et al., 2019), 33.3% in Belgium (Baert et al., 2011). HAV shows higher percentage during this study than HNoV, Approximately 50% of the Egyptian population has already been exposed to the HAV by the age of 15, and in rural areas of Egypt, the prevalence of HAV reaches 100% (Meky et al., 2006). To examine the effect of handler of field-workers on the transportation of these viruses by fresh produce or by strawberry to the public peoples, more studies need to be done to prove this route of contamination. Huvarova and co-workers traced the presence of NoVs in vegetables and herbs samples that were collected from markets and farms. NoVs (including GI and GII) were detected in 3.2% and 1.2% from markets and farms, respectively in addition to the presence of these viruses on the hands and gloves of workers (Huvarova et al., 2018).

Conclusion

The findings of this study underline the significance of a viral surveillance program for soft fruits and fresh vegetables. We anticipate that our research will aid in the management and control of microbiological risks in fresh food, lowering the risk associated with consuming contaminated foods.

Acknowledgements Not applicable

Data Availability The authors declare that all data supporting the findings of this study are available within the article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest that could inappropriately influence this work.

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