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Characterization of Enteroviruses in Irrigating Water in Egypt and in Clinical Samples with the Assessment of Ethanolic Extract of *Nigella sativa* in Response to these Viruses

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A B S T R A C T

This study aimed to determine enteroviruses using different PCR methods and also studying the effect of ethanolic extract of Nigella Sativa on isolated viruses in vitro. we performed reverse transcription-PCR (RT-PCR), nested PCR and cell culture-PCR followed by real time PCR to monitor the occurrence of enteric viruses in in 23 samples collected from irrigation water sites in El-Giza and El-Menofya Governorates from March 2017 to May 2017; fourteen samples from five rural sites in El-Menofya which no sanitation systems service these regions and nine samples from three urban sites in El-Giza served by sanitation systems were investigated. The comparison of the detection techniques was involved in study. The highly detected virus was Adenoviruses followed by rotaviruses then Noroviruses. Presence of sanitation systems may affect the frequency of enteric viruses in irrigation water. Rotaviruses were the most frequent viruses isolated from the diarrheal samples followed by adenoviruses and finally noroviruses. Considerable reduction of genome copies and infectious units of both rotavirus Wa strain and adenovirus type 7 was observed by treatment with ethanolic extract of Nigella Sativa *in vitro*.

Introduction

Water is playing a powerful role in the transmission of different types of viruses. Fecal oral route is the main cause of transmitting enteric viruses to people or which called waterborne viral diseases. Patients suffering from gastroenteritis or hepatitis may excrete from 105 to 1011 virus particles per gram of stool, Monsalvo (2016). Usually these viruses have been found in groundwater, sewage, rivers, irrigating systems and even in treated drinking water and they are recognized as major public health hazards.

It was found that these viruses had not been removed or inactivated by procedures taken for treatment water. Part of the problems that we facing with viruses in treated water and raw wastewater lies in the distribution systems and the failure to ensure the complete removal of viral pathogens consequently, viruses become environmental pollutants and the solid-associated viruses in wastewater effluents are discharged into aquatic environments and accumulate in the sediments where they persist longer than in the water column (Kovac *et al.,* 2009) As a matter of fact, sediments act as a The fate of microbial enteric pathogens may take many potential routes in the water environment. Humans are exposed to enteric viruses through various routes: shellfish grown in contaminated waters, food crops grown in land irrigated with wastewater and/or fertilized with sewage, sewage-polluted recreational waters and contaminated drinking water. Usually, waterborne infections are acquired through the ingestion of contaminated water or shellfish, Pepper *et al.,* (2006).

Rotavirus is a member of family reoviridea composed of segmented double strained RNA and triple layer capsid. Group A rotavirus is responsible for 90% of clinical disease of rotavirus Acip *et al.,* (2009). The rotavirus is considered as the common cause of gastroenteritis in children less than 5 years old around the world, in 2013, WHO estimated the deaths caused by rotavirus infection in children less than 5 years annually the result was 215.000 children and more than 2 million children hospitalized with dehydration. After application of rotavirus vaccine (RotaTeq and Rotarix) in industrial and low resource countries the hospitalization decreased by 90% (Acip *et al.*, 2009). If the infection occurs even one time in life will give 85% immunity toward severe gastroenteritis, 75% immunity toward any rotavirus gastroenteritis and 35% immunity toward any infection of rotavirus. (Bernstein 2009). In Europe, many countries record different rates of rotavirus infection and hospitalized cases. The rate of hospitalized children aged less than 5 years is changing in Denmark from 3.8/1000 per year to 12.8 in Ireland. In 2004, the rotavirus turn to modifiable disease in Ireland, so from this time the rising in notification of rotavirus disease become sustained especially in children aged less than 4 years (September, 2016).

Human adenovirus (HAdV) is a double stranded DNA, non-enveloped virus that belong to family Adenoviridea with 7 species from A to G, 70 distinct genotypes and 50 serotypes that numbered from 1 to 50. There is many different symptoms of adenovirus infection according to the site of replication of each species for example upper or lower respiratory tract human adenovirus infection caused by species B, C and E, eye conjunctiva caused by species B and D and gastrointestinal tract infection occur by F and G species. Occurrence of human Adenovirus outbreaks usually in closed population such as military instillation and hospitals. In Egypt: Eighty-eight adenovirus (Ad) isolates and associated clinical data were collected from walk-in patients with influenza-like illness in Egypt during routine influenza surveillance from 1999 through 2002, Metzgar *et al.*,(2005).Respiratory Adenovirus distributions are geographically variable, and serotype prevalence has not been previously characterized in this region. (Metzgar, 2005)

Norovirus is a positive non-enveloped single stranded RNA virus that belongs to family Caliciviridea Norovirus divided into 5 genotypes from GI to GV and the strains that can infect human are in GI, GII and GIV, Richards, *et al.,* (2004) Scipioni *et al.*,(2008). Norovirus cause gastroenteritis in children and adults. It can be transmitted from person to another person through fecal oral route or

droplets of vomits. Stable in environment. Low dose of viral particles (≥18) can cause infection. It has the ability to stay alive in high and low temperature and has a resistance against many chemical disinfectants Andrew *et al.*,(2012). It is common in winter so it called winter vomiting disease. Symptoms of infection are nausea, vomiting, headache, diarrhea, dizziness and stomach pain. Norovirus consider the most common cause of viral gastroenteritis and major cause of outbreaks in closed populations such as vacation settings, schools, nursing homes and hospitals. (franck,2014).

In Cairo, Egypt, rotavirus (Group A) is the most frequent RNA enteric viruses in raw sewage and Nile water and also the most resistant RNA enteric viruses to treatment processes in water and wastewater treatment followed by HAV, astrovirus and enterovirus and finally, noroviruses respectively (Villena *et al.,* 2003; El-Senousy *et al.,* 2004; El-Senousy *et al.,* 2007; Pinto *et al.,* 2007; El-Senousy *et al.,* 2013a; El-Senousy *et al.,* 2013c). In Delta, Egypt, rotavirus has high prevalence in water and wastewater (El-Senousy and El-Mahdy 2009; El-Senousy *et al.,* 2013c) while norovirus has low prevalence in Egyptian naturally contaminated irrigation water and fresh produce (El-Senousy *et al.,* 2013b). Improvement of sensitivity, specificity, and validation of methods for detection and quantification of norovirus in Egyptian irrigation water and fresh produce was done (El-Senousy *et al.,* 2013b) and the differentiation between infectious and non infectious astroviruses in Egyptian sewage and water samples was also done (El-Senousy *et al.,* 2007). In the study of El-Senousy and Co-workers (2013a) high prevalence of human rotavirus and adenovirus in sewage and water samples before and after treatment steps and also in clinical samples was reported. Adenovirus had a little higher frequent than rotavirus and it was detected all the year while rotavirus ha its peak in autumn and winter. El-Senousy and Abou El-Ela (2017) suggested high efficiency of small pilot wastewater treatment plant in viral removal from Egyptian raw sewage. Another study was reported for efficient microbial removal in Egyptian sewage (Tawfik et al, 2012). A lot of trials to discover antiviral drug (using natural products or synthetic compounds) or vaccine depending on the frequent Egyptian strain were reported (Abdo et al, 2012, Ateya et al, 2016, Bassyouni et al, 2012,, El-Baz et al, 2013, El-Senousy et al, 2013c, Essawy et al, 2011, Hamdy, N. and El-Senousy, 2013, Hamdy, N. et al, 2013,, Matloub et al, 2015, Matloub et al, 2017, Rashad et al, 2015,) *Nigella sativa* Linn (Ranunculaceae) is popularly known as black cumin with a wide spectrum of pharmacological activities including anti-inflammatory, antibacterial, antifungal and antihelmenthic (Dwarampudi et al, 2012).

The objective of this study was to investigate rotaviruses, adenoviruses, and noroviruses in the irrigation water from different parts in rural and urban Egyptian regions. Also, to study the effect of *Nigella sativa* on rotaviruses and adenoviruses depending on the mechanism of blocking the host receptors *in vitro*. reservoir from which viruses are re-suspended in the water column by several natural or artificial phenomena (Hassard *et al.,* 2016).

Materials and Methods

Samples

Collection

23 samples (Forteen samples from five sites in El-Menofya which no sanitation systems serve these regions and nine samples from three sites in El-Giza served by sanitation systems were investigated.) were collected from irrigation water in different sites in El-Giza

Governorate and El-Salamlek, Aboud, Koshk Galila, Bawabat, and Asroma sites in El-Menofya Governorates during the period from March 2017 to May 2017. Ten liters from each sample were collected and transferred to the laboratory to be concentrated in the same day.

Diarrheal samples from children 0-5 years were collected from governmental hospitals serve the region where the environmental samples collected (100 samples from El-Giza Governorate and 100 samples from El-Menofya Governorate)

Plant materials

Nigella sativa seeds were collected from Egyptian market in Cairo in July 2017.

Processing of the samples

Concentration of irrigation water samples

Samples were concentrated by filtration through negatively charged nitrocellulose membranes (0.45 µm pore size and 142 mm diameter) after addition of AlCl3 to a final concentration of 0.5 mM and acidification to pH 3.5. The viruses adsorbed to the membrane were eluted with 75 ml of 0.05 M glycine buffer, pH 9.5 containing 3% beef extract (Lab-Limco powder, Oxoid; Smith and Gerba, 1982; Rose et al, 1984). All samples were deconcentrated using an organic flocculation method (Katzenelson et al, 1976). Briefly, the eluate was acidified to pH 3.5 using HCl (5N) and centrifuged at 3000 rpm for 15 minutes, the supernatant was discarded and the pellet was dissolved in 1 ml and 5 ml of Na_2HPO_4 (0.14N, pH 9).

Concentration of clinical samples

Approximately 0.1 g of stool samples was weighed, diluted 1:10 in nuclease- free H2 0 and vortexes for 30 sec. Samples were clarified by centrifugation at 7.000 rpm for 10 min at room temperature. Viral RNA was extracted from 140 µl of the supernatant.

Ethanoic extraction of *Nigella sativa*

The *Nigella sativa* seeds were washed with water to remove dust particles, Shade dried and extracted by boiling 500 g of the seed powder (twice) in 3000 mL of 95% Ethanol for 30 min at 70°C according to Dwarampudi and co-workers (2012).

Viral nucleic acid extraction

Viral RNA was extracted from 140 µl of the supernatant using BIOZOL Total RNA Extraction reagent (BIOFLUX—Japan) and according to the manufacturer's instructions and to a 30 µl final volume.

RT-PCR of a Fragment of the VP6-Coding Gene of Rotaviruses Group A

The primers used for RT-PCR were the forward VP6-F 5-GATGGATCNACTACATAGT-3 and the reverse VP6-R 5-GTCCGGTTCATAGGTCCTGG-3 primers (0.5 µm for each), and according to Iturriza-Gomara and co-workers (2002) using 100 U of M-MLV reverse transcriptase enzyme (Promega—USA) in a total volume of 10 µl and 1.5 U of Taq DNA polymerase (Biobasic—Canada) in a total volume of 50 µl. Nested PCR amplification of the target rotavirus VP6 fragment was performed using the forward primer, VP6-NF 5-GCTAGTTTAAGGGATACA-3, and the reverse primer, VP6-NR 5-TCTATAGCCCGTTAATC-3 (1 µm for each), and according to Gallimore and co-workers (2006) **to** amplify 155 bp fragment. PCR products (10 µl) were analyzed by electrophoresis on 3 % agarose gels (Panreac—Spain).

Quantification of rotavirus group A genome copies using real-time RT-PCR method

Real-time TaqMan RT-PCR was performed for positive samples in the previous RT-PCR screening. Real-time PCR was done using rotavirus@ceeramTools™ Food & Environmental kit and according to manufacturer's instructions using Rotavirus - Q Standard (Ceeram Tools).

Cell culture RT-PCR (CC-RT-PCR) for quantification of infectious rotavirus particles

Rotavirus CC-RT-PCR assay was performed according to Abad and co-workers (1997); El-Senousy and co-workers (2007); and Ghazy and co-workers (2008). The assay was performed on suspensions of infected MA104 cells. Primers VP6-F and VP6-R were used. The RT-PCR method was the same as described previously. The detection limit in this tissue culture assay using 100 µl of inoculum is 1X101 CC-RT-PCR units/ml, where CC-RT-PCR unit is the reciprocal endpoint dilution detectable by CC-RT-PCR.

RT-PCR for the detection of norovirus

Viral RNA of the capsid gene was amplified using the RT-PCR method according to kageyama and co-workers (2003) and kojima and co-workers (2002). Ten microliters from the nested- PCR were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The lengths of the products from the nested-PCR were 330 bp for norovirus GGI and 344 bp for norovirus GGII.

Molecular assays for human norovirus genome copies quantification

A standardized one-step real-time TaqMan RT-PCR using previously described primers and probes was employed for the detection of human noroviruses GGI (da Silva *et al.,* 2007; Svraka *et al.,* 2007) and GGII (Kageyama *et al.,* 2003; Loisy *et al.,* 2005) in 5 µl of nucleic acid extracts. Quantification through a real-time procedure following the same conditions as for noroviruses with previously described primers and probes (Pintó *et al.,* 2009) was done.

Extraction of DNA

It was done as described previously by Kapperud and co-workers (1993) and modified by Estrada *et al.,* (2007). Fifty µl of sample concentrate were added to 50 µl of 1X PCR buffer containing 0.2mg of Proteinase K/ml. After being incubated at 37°C for 1h, the suspension was boiled for 10 min and then centrifuged at 12500 rpm for 5 min at 4°C. The supernatant was used for performing the PCR.

Detection of adenoviruses using nested PCR

It was done according to Puig and co-workers (1994) using the specific primers hex AA 1885, hex AA 1913 for the first round PCR and nehex AA 1893 and nehex AA 1905 for the second round PCR for detection of human adenovirus and were selected from the DNA sequence of the open reading frame of hexon gene. PCR products (10 µl) were analyzed by electrophoresis on 3% agarose gels (Panreac-Spain).

Real-Time PCR for quantification of adenoviruses

Real-time TaqMan PCR was performed for positive samples in the previous PCR screening. Real-time PCR was done using adenovirus@ceeramTools™ Food & Environmental kit and according to manufacturer's instructions using adenovirus - Q Standard (Ceeram Tools) and using a real-

time PCR thermal cycler (Rotor-Gene Q, Qiagen).

Cell culture-PCR (CC-PCR) technique for quantification of adenovirus infectious units

It was done according to Esawy and co-workers (2011) and Abdo and co-workers (2012) Adenovirus cell culture-PCR (CC-PCR) assay was performed on suspensions of the infected Hep-2 cell line. Set of primers, hex AA 1885and hex AA 1913 was used. The detection limit in this tissue culture assay using 100 µl of inoculum is 1x101 CC- PCR units/ml (u/ml), An adenovirus CC-PCR unit is defined as the reciprocal endpoint dilution detectable by CC- PCR.

Cytotoxicity test

It was done according to Simoes, etal., (1999) and Walum, Strenberg, and Jenssen (1990). Briefly, decontamination of samples was done by adding 24 µL of 100× of antibiotic–antimycotic mixture to 1 ML of the sample. Then, bi-fold dilutions were done to 100 _µL of original dissolved samples and 100 _µL of each dilutions were inoculated in MA104 and Hep-2 cell lines (obtained from the Holding Company for Biological Products & Vaccines VACSERA, Egypt) previously cultured in 96 multi well plates (Greiner-Bio one, Germany) to estimate the non toxic dose of the tested samples. Cytotoxicity assay was done using cell morphology evaluation by inverted light microscope and cell viability test applying trypan blue dye exclusion method.

Cell morphology evaluation by inverted light microscopy

MA104, and Hep-2 cell cultures (2×10^5 cells/ml) were prepared separately in 96- well tissue culture plates (Greiner-Bio one, Germany). After 24 h incubation at 37 ˚C in a humidified 5% (v/v) CO2 atmosphere cell monolayers were confluent, the medium was removed from each well and replenished with 100 _µL of bi-fold dilutions of different samples tested prepared in DMEM (GIBCO BRL). For cell controls 100 _µL of DMEM without samples was added. All cultures were incubated at 37 ˚C in a humidified 5% (v/v) CO_2 atmosphere for 72 h. Cell morphology was observed daily for microscopically detectable morphological alterations, such as loss of confluence, cell rounding and shrinking, and cytoplasm granulation and vacuolization. Morphological changes were scored (Simoes *et al.,* 1999).

Cell viability assay

It was done by trypan blue dye exclusion method (Walum *et al.*,1990). MA104, and Hep-2 cell cultures (2×10^5 cells/ml) were grown in 12-well tissue culture plates (Greiner-Bio one, Germany). After 24 h incubation, the same assay described above for tested samples cytotoxicity was followed by applying 100 µL of tested samples dilutions (bi-fold dilutions) per well. After 72 h the medium was removed, cells were trypsinized and an equal volume of 0.4% (w/v). Trypan blue dye aqueous solution was added to cell suspension. Viable cells were counted under the phase contrast microscope.

Determination of Rotavirus Wa strain, and Adenovirus type 7 Titers Using plaque assay

The mixture was incubated for 1/2 hr in 37°C. The non-toxic doses of the ethanolic extract of *Nigella sativa* was inoculated directly in MA-104 and Hep-2 cell lines and after 1 hr incubation in 37°C addition of maintenance media was performed. After 24 hours incubation, the inoculation of (100µl) 10 fold dilutions of 100µl of different doses of rotavirus Wa strain, and adenovirus type 7 ($1X10^5$, $1X10^6$, $1X10^7$) was carried out

separately into MA104, and Hep-2 cell lines in 12 multi well- plates. The infectivity of the rotavirus stocks were activated with 10 µg/ml trypsin for 30 min at 37°C. After 1 hr of incubation for adsorption at 37°C in a 5% CO2-water vapor atmosphere without constant rocking. The plates were rocked intermittently to keep the cells from drying.

After adsorption, 1 mL of 2X media (Dulbecco´s Modified Eagle Medium, Gibco-BRL (DMEM) plus 1ml 1% agarose was added to each well, 0.5 µg/ml was added to the media-agarose mixture in the case of rotavirus Wa strain and the plates were incubated at 37°C in a 5% CO2-water vapor atmosphere. After the appropriate incubation period, the cells were stained with 0.4% crystal violet after formalin fixation, and the number of plaques counted. The viral titers were then calculated, and expressed as plaque-forming units per milliliter (PFU/ml) (Schmidtke et al, 1998).

Results and Discussion

Detection of rotaviruses, noroviruses, and adenoviruses in irrigation water samples

From areas with no sanitation systems service (Menofya sites)

Adenoviruses were detected in 7/14 (50%) Rotaviruses were detected in 5/14 (35.7%) in Noroviruses were detected in 2/14 (14.3%)

From areas with sanitation systems service (Giza sites)

Adenoviruses were detected 2/9 (22.2%) Rotaviruses in 1/9 (11.1%). Noroviruses did not detected 0/9 (0%), table (1)

Through Analysis of the irrigation water in all samples using Nested PCR method; Adenoviruses were detected in 9/23 samples, rotaviruses were detected in 6/23 samples while noroviruses were detected in 2/23 samples (fig.1).

Quantification of the infectious units of the investigated viruses in the positive irrigation water samples

Indicated that rotavirus infectious units 8X10 to $9X10^3$ infectious units/litre, adenovirus infectious units were $1X10^2$ infectious units/litre in all investigated samples. Some samples showed no infectivity for Rotavirus, although the viral genome was detected in these samples, table (2).

Seasonal detection of viruses in the 23 analyzed irrigation water

Quantification of genome copies of the investigated viruses in the positive irrigation water samples indicated that rotavirus genome copies ranged from $6X10^2$ to $6X10^3$ genome copies/litre, adenovirus genome copies ranged from $2X10^4$ to $9X10^4$ genome copies/litre, and noroviruses genome copies ranged from $1X10^2$ to $2X10^2$ genome copies/litre Table (2). No difference was observed between rural and urban regions

Frequency of enteric viruses in diarrheal samples

The frequencies of Rotaviruses, Adenoviruses, and Noroviruses were 35%, 22%, and 8% respectively in clinical specimens of El-Menofya, while they were

27%, 19%, and 4% respectively in clinical specimens of El-Giza Table (3).

Estimation of ethanolic extract of *Nigella sativa* activity against the examined viruses

The non-toxic doses of ethanolic extract of *Nigella sativa* were 130 µg/ml and 120 µg/ml

in MA-104 and Hep-2 cell lines respectively. The reductions of rotavirus Wa strain and adenovirus type 7 were 33.3% and 26.7% respectively when inoculated in MA-104 and Hep-2 cell lines previously treated with the non-toxic doses of ethanolic extract of *Nigella sativa* Tables (4 and 5).

About 3.4 million people especially children die every year as a result of diseases related to water. Absence of treatment in different sources of used water led for producing human enteric viruses. There are more than 100 viral species that are the main cause of infection to human.

Among the many viruses discharged are such pathogens as Rotavirus, Norovirus, and Adenovirus. The significance of these viruses in human range between gastrointestinal, bronchitis and conjunctivitis. Most of these agents transmitted via fecal oral rout or person to person contact. Irrigation water is one of the main sources of enteric viruses transmission to human and animals.

Table.1 Detection of viruses in all 23 irrigating water specimens (with and without sanitation systems service) of the different regions examined

Samples	Collection date	Rota viruses	Noro virus	Adeno viruses
El-Salamlek	14/3/2017	+	-	+
Aboud	14/3/2017	-	-	-
Koshk Galila	14/3/2017	+	+	+
Giza site 1	14/3/2017	-	-	-
Giza site 2	14/3/2017	+	-	+
Giza site 3	14/3/2017	-	-	-
El-Salamlek	22/3/2017	-	-	-
Aboud	22/3/2017	-	-	-
Koshk Galila	22/3/2017	+	-	+
Giza site 1	22/3/2017	-	-	-
Giza site 2	22/3/2017	-	-	+
Giza site 3	4/2017	-	-	-
El-Salamlek	4/2017	-	-	-
Aboud	4/2017	+	-	+
Koshk Galila	4/2017	-	-	+
Giza site 1	4/2017	-	-	-
Giza site 2	4/2017	-	-	-
Giza site 3	4/2017	-	-	-
El-Salamlek	5/2017	-	-	-
Aboud	5/2017	-	-	-
Koshk Galila	5/2017	-	-	-
Bawabat	5/2017	-	-	+
Asroma	5/2017	+	+	+

Table.2 Quantification of Rotaviruses, Norovirus and Adenoviruses infectious units/liter in irrigation water samples

Samples	Date	Rota viruses	Noro viruses	Adeno viruses
El-Salamlek	14/3/2017	$6X10^3$	-	**$8X10^4$**
Aboud	4/3/2017	-	-	-
Koshk Galila	14/3/2017	$4X10^3$	$2X10^2$	**$5X10^4$**
Giza site 1	14/3/2017	-	-	-
Giza site 2	14/3/2017	$1X10^3$	-	**$2X10^4$**
Giza site 3	14/3/2017	-	-	-
El-Salamlek	22/3/2017	-	-	-
Aboud	22/3/2017	-	-	-
Koshk Galila	22/3/2017	$3X10^3$	-	**$9X10^4$**
Giza site 1	22/3/2017	-	-	-
Giza site 2	22/3/2017	-	-	**$8X10^4$**
Giza site 3	4/2017	-	-	-
El-Salamlek	4/2017	-	-	-
Aboud	4/2017	$1X10^3$	-	**$9X10^4$**
Koshk Galila	4/2017	-	-	**$2X10^4$**
Giza site 1	4/2017	-	-	-
Giza site 2	4/2017	-	-	-
Giza site 3	4/2017	-	-	-
El-Salamlek	5/2017	-	-	-
Aboud	5/2017	-	-	-
Koshk Galila	5/2017	-	-	-
Bawabat	5/2017	-	-	**$2X10^4$**
Asroma	5/2017	$6X10^2$	$1X10^2$	**$9X10^4$**

Table.3 Frequency of enteric viruses in clinical samples of children

Type of virus	Frequency and percentage of viral frequency in clinical specimens	
	In El-Menofya	In El-Giza
Rotaviruses	35/100 (35%)	27/100 (27%)
Adenoviruses	22/100 (22%)	19/100 (19%)
Noroviruses	8/100 (8%)	4/100 (4%)

Table.4 Antiviral activity of non toxic doses of tested materials against rotavirus Wa strain

Tested materials	Initial Viral titre	Final viral titre	% of reduction	Mean % of reduction
Ethanolic extract of *Nigella sativa*	$1X10^5$	$6X10^4$	40%	**33.3%**
	$1X10^6$	$7X10^5$	30%	
	$1x10^7$	$7x10^6$	30%	

Table.5 Antiviral activity of non toxic doses of tested materials against adenovirus type 7

Tested materials	Initial Viral titre	Final viral titre	% of reduction	Mean % of reduction
Ethanolic extract of *Nigella sativa*	$1X10^5$	$7X10^4$	30%	**26.7%**
	$1X10^6$	$7X10^5$	30%	
	$1x10^7$	$8x10^6$	20%	

Figure.1 Detection of viruses in all 23 irrigating water specimens (with and without sanitation systems service) of the different regions examined

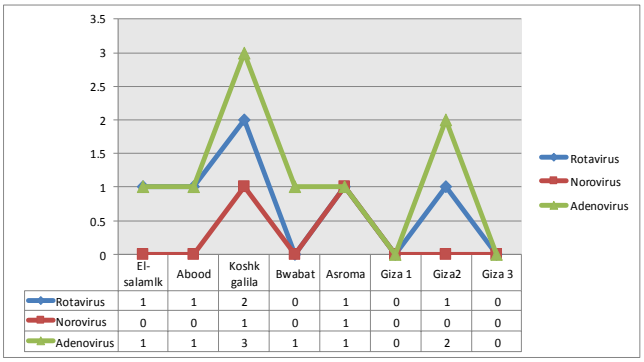
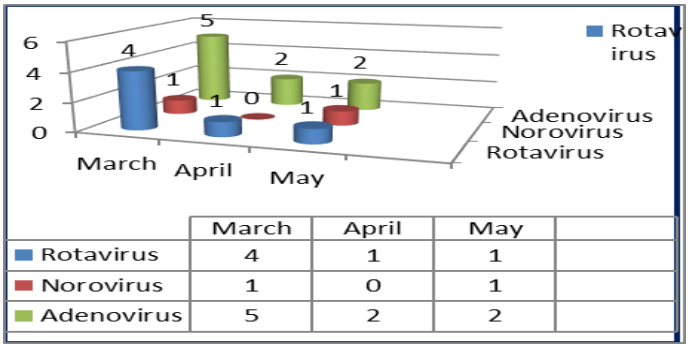


Fig.2 Seasonal detection of viruses in 23 irrigating water specimens from the different regions examined



Our results indicated that of enteric viruses investigated in irrigation water; Adenoviruses were the most frequent followed by Rotaviruses, and finally Noroviruses. This pattern was observed in samples collected from either Menofya and Giza sites These results are comparable with the pattern detected in ground water used for irrigation in Giza Governorate (El-Senousy et al, 2013d).. On the other hand, the frequency of viruses in irrigation water samples collected from Menofya is higher than the viral frequency in Giza irrigation water samples. This pattern is clear for all the studied viruses. The higher frequency of viruses examined in Menofya irrigation water samples than Giza irrigation water samples may return to the absence of wastewater treatment systems in the sampling region of Menofya. As there were wastewater treatment systems serve the sampling regions of Giza. Although, Kokkinos *et al.*, (2011)and Hewitt *et al.*, (2013) reported that Adenoviruses are frequently detected in high concentrations in treated wastewater and wastewater-contaminated waters., Allard and Vantarakis, (2017) concluded that Adenovirus concentrations ranged from 1 to about 100,000 genome copies per litre of surface water in 8 studies made in 5 different countries.

Qualitative analysis is not enough to ensure the health hazard, so quantitative analysis for both genome copies and infectious units was used. Again, our results showed that the number of genome copies of Adenoviruses was higher than Rotaviruses. Noroviruses were less than the other both viruses in the number of genome copies. The number of Noroviruses quantified in our study is closely related to the number quantified in irrigation water samples collected previously from Egyptian Delta region (El-Senousy et al, 2013b). El-Senousy and co-workers (2007) confirmed the relationship between the number of genome copies and infectious units. Our article gives a new evidence for the confirmation of this direct relationship. The number of infectious units of Noroviruses could not be quantified because till now, there is no cell line suitable for propagation of Noroviruses.

Several investigators have used different methods to detect Adenoviruses in various environmental samples including water, Calgua *et al.*, (2013) and Ahmed *et al.*, (2015) based on PCR procedures. Cell cultures techniques and cell culture techniques combined with PCR have also been used by Ko *et al.*, 2003; 2005). Several investigators have used different methods to detect Adenoviruses in various environmental samples including water.

The frequency pattern in irrigation water was different from the pattern observed in diarrheal samples; Rotavirus was the most frequent virus. Rotavirus is the first viral causative agent for gastroenteritis worldwide followed by Adenovirus. Tate *et al.*, (2010) showed that Rotavirus (RV), Adenovirus (AdV) and Norovirus (NoV) are often related with both diarrhea and subclinical infections;. They also reported that these viruses shed in the feces of infected (symptomatic and asymptomatic) individuals, and transmitted through the fecal-oral route, Barardi *et al.*, (2012).

On the contrary, Aadenovirus is the most frequent virus in the environmental samples. It may return to the higher stability of Adenovirus against the environmental conditions like temperature and pH. It gives higher survival rate for Adenovirus (DNA genome) than Rotavirus (RNA genome). Also, Adenovirus has not a peak of seasonal variation, while Rotavirus has a peak in autumn and winter. It gives a chance to Adenovirus to be detected in the environmental samples all the year. Nucleic

acid amplification methods was used for recognition and detection of the most common enteroviruses; Noroviruses, Rotavirus and human Adenoviruses in the irrigating water from 3 areas in EL-Giza and 5 areas in El-Monofia which in order to screen the viral strains. That is always circulating in different communities. This statement was confirmed by Barardi *et al.*, (2012) and mentioned that these enteric viruses have a special concern in public health due to resistance under environmental conditions.

Shellfish that grown in contaminated waters, food crops grown in land irrigated with wastewater and/or fertilized with sewage, sewage-polluted recreational waters and contaminated drinking water are suitable condition for polluted these sources of water by these viruses. Usually, waterborne infections are acquired through the ingestion of contaminated water or shellfish. The feature or the thyme of the study was the application of such detection which provides important data on the frequency, distribution, and spread of these viruses in a period of time from March to May which support epidemiological studies of the related viral infections. The seasonal variation on the shedding of Adenovirus seems to be dependent on the geographic region analyzed and the presence of clinical manifestations. Hamedi *et al.*, (2010).

The reductions of Rrotavirus Wa strain and Adenovirus type 7 were 33.3% and 26.7% respectively when inoculated in MA-104 and Hep-2 cell lines previously treated with the non-toxic doses of ethanolic extract of *Nigella sativa*. The pre-inoculation of the extracts may lead to block the receptors specific for both viruses in the two specific cell lines (host systems). This mechanism differs from the other mechanisms which depend on the direct action on the virus or stop the replication cycle, Adel Abdel-Moneim *et al.*,(2013) *concluded that Nigella sativa* ethanolic extracts to HCV patients exhibited potential therapeutic benefits *via* decreasing viral load and alleviating the altered liver function,. Although, the percentage of reduction is not so high (less than 50%). it may be promising according to the used mechanism (blocking the receptors specific for viruses). Using of the natural products in regular way or as additives to some daily used food may lead to some protection against gastroenteritis causing viruses.

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