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Survival of Adenovirus, Rotavirus, Hepatitis A Virus, Pathogenic Bacteria and Bacterial Indicators in Ground Water

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Abstract: The aim of this work is to study the survival of genome and infectious units of adenovirus type 40, rotavirus Wa strain and Hepatitis A virus (HAV) HM 175 adapted strain in Egyptian ground water at different temperatures and their relationships with the persistence of bacterial indicators and pathogenic bacteria. Also, study the effect of limestone on the same viruses and bacteria in ground water. The genome of adenovirus was the more stable and persistent followed by rotavirus Wa strain and finally HAV HM 175 adapted strain. There were gradual decreases in the genome of the three tested viruses parallel with the time. After 12 weeks, adenovirus type 40 genome copies reductions were 0.05 $\log_{10}$, 0.5 $\log_{10}$ and 1.5 $\log_{10}$ at 4°C, 22°C and 35°C respectively. Rotavirus genome copies reductions were 0.1 $\log_{10}$, 0.7 $\log_{10}$ and 2.3 $\log_{10}$ at 4°C 22°C and 35°C, respectively. HAV genome copies reductions were 0.2 $\log_{10}$, 0.8 $\log_{10}$ and 2.7 $\log_{10}$ at 4°C, 22°C and 35°C, respectively. The infectious units of adenovirus were the more stable and persistent than rotavirus Wa strain. There were gradual decreases in the infectious units of the two tested viruses parallel with the time. After 12 weeks, adenovirus infectious units reductions were 0.5 $\log_{10}$, 1.5 $\log_{10}$ and 3 $\log_{10}$ at 4°C, 22°C and 35°C, respectively Rotavirus infectious units reductions were 0.7 $\log_{10}$, 1.8 $\log_{10}$ and 3.5 $\log_{10}$ at 4°C, 22°C and 35°C, respectively. Higher stability for the genomes of the three tested viruses and the infectious units of adenovirus and rotavirus than *E. coli*, *Enterococcus fecalis, Salmonella spp*. and *Staphylococcus aureus* in ground water at 22°C after 12 weeks was observed. The results showed that, there were gradual decreases. One $\log_{10}$ reduction was recorded after 5 weeks for *E coli*, *Salmonella spp.* after 8 weeks for *Staphylococcus aureus* and after 9 weeks for *Enterococcus fecalis* when all of them were seeded separately. On the other hand bacterial indicators (*E. coli* and *Enterococcus fecalis*) had more survival than pathogenic bacteria (*Salmonella Spp.* and *Staphylococcus aureus*). The persistence of viruses and bacterial indicators as well as pathogenic bacteria in ground water treated with limestone was also studied. After 96 hours, the mean reductions of genome copies and infectious units were 0.9 $\log_{10}$, 1.2 $\log_{10}$ and 1.4 $\log_{10}$ for adenovirus type 40, rotavirus Wa strain and HAV HM 175 adapted strain genome copies, respectively and 1.8 $\log_{10}$ and 2.3 $\log_{10}$ for adenovirus type 40 and rotavirus Wa strain infectious units respectively in ground water treated with limestone (6.7 g/l). Also, the results showed that, decrease one log (removal 90%) in flask which contained *Salmonella spp*. and *E. coli* treated with limestone was observed after 2 and 4 hours, respectively, the effect of limestone on bacterial indicators and pathogens was faster and higher than the effect on viral genome and infectious units.

Key words: Adenovirus · *Enterococcus fecalis* · *E. coli* · Hepatitis A virus · Limestone · Ground water · Rotavirus · *Salmonella spp.* · *Staphylococcus aureus*

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INTRODUCTION

Groundwater is an important source of drinking water in many regions of the world. Groundwater constitutes 95 percent of the freshwater that is suitable for human consumption [1]. Groundwater is considered to have a more stable composition and a higher microbial quality than those of surface waters. This is partly due to the slow filtration of the water through layers of soil and sediments, removing pathogenic microorganisms and many chemical compounds [2]. The role of groundwater as a source of outbreaks in different countries was recorded. Among waterborne pathogens bacteria, enteric viruses seem to have a high potential to reach aquifers and the genome of enteroviruses, noroviruses, rotaviruses, or hepatitis A virus had been detected in many groundwater supplies [3-5]. The presence of enteric viruses may be explained by their physical and chemical characteristics. These viruses are extremely small (20 to 100 nm), readily passing through sediment pores that trap larger pathogens such as bacteria and protozoa [6]. Some viruses also show surface properties that allow very high rates of diffusion through soils [7, 8]. Another explanation is linked to the survival of viral particles. Infectious enteric viruses commonly persist longer in the environment than do fecal bacteria [9]. Further, the viral genome has a longer persistence than does an infectious particle [10].

One of the most important characteristics of pathogenic bacteria which influenced water resources is their survival ability under natural conditions. The survival of bacteria in environment depends on several factors including bacterial species, competition with other microorganisms [11]. The organic compounds essential for the survival of heterotrophic bacteria in groundwater mainly originate from secondary organic compounds that percolate from the surface and their quantity and quality depend directly on the reloading rate of the water table and on the leaching of soil organic matter [12]. Oitt *et al.* [13] reported that, pathogenic bacteria were present in natural water for several months with absence of bacterial indictors. In addition, in France, Grisey *et al.* [14] reported that, total coliforms, *Escherichia coli*, Enterococci, *Pseudomonas aeruginosa, Salmonella spp.* and *Staphylococcus aureus* were monitored for 15 months in groundwater. Furthermore, Gözdereliler [15] concluded that bacteria are adapted to low substrate concentrations present in ground water. Hydrated lime is widely used to adjust the pH of water to prepare it for further treatment. Lime is also used to combat "red water" by neutralizing the acid water, thereby reducing corrosion of pipes and mains from acid waters. The corrosive waters contain excessive amounts of carbon dioxide. Lime precipitates the CO_2 to form calcium carbonate, which provides a protective coating on the inside of water mains. Lime is used in conjunction with alum or iron salts for coagulating suspended solids incident to the removal of turbidity from "raw" water. It serves to maintain the proper pH for most satisfactory coagulation conditions. In some water treatment plants, alum sludge is treated with lime to facilitate sludge thickening on pressure filters. By raising the pH of water through the addition of lime and retaining the water in contact with lime for 24-72 hours, lime controls the environment required for the growth of bacteria and certain viruses. This application of lime is utilized where "phenolic water" exists, because chlorine treatment tends to produce unpalatable water due to the phenol present. This process, called "excess alkalinity treatment," also removes most heavy metals [16]. Sepehrnia *et al.* [11] demonstrated that, calcium carbonate compound had an effect on survival of *E. coli* in geological area of Iran, which can decrease filtration costs. Furthermore, Mara and Johnson [17], used aerated filter from lime rock ($CaCO_3$) for the removal of fecal coliform from outlet of wastewater treatment plant at Esholt, Bradford, England, they found reduction about 99% from initial counts in the final effluent. The aim of this study is to evaluate the survival and persistence of genome and infectious units of adenovirus type 40, rotavirus Wa strain and HAV HM 175 adapted strain in Egyptian ground water at different temperatures and their relationships with the persistence of bacterial indicators and pathogenic bacteria. Also, study the effect of limestone on the same viruses and bacteria in ground water.

MATERIALS AND METHODS

Viruses and Cell Lines: Rotavirus Wa strain and HAV HM175 adapted strain provided by Prof. Dr. Albert Bosch, University of Barcelona, Spain. Adenovirus type 40 provided by Dr. Ali Fahmi (The Holding Company for Biological Products and Vaccines VACSERA, Cairo, Egypt). Hep-2 and MA104 cell lines were also provided by VACSERA, Cairo, Egypt.

Determination of the Persistence of Adenovirus Type 40, Rotavirus Wa Strain and HAV HM 175 Adapted Strain in Ground Water at Different Temperatures: Three doses of each virus genome quantified before ($1X10^3$, $1X10^5$,

$1X10^7$) genome copies were seeded separately in 1L of ground water taken from well water in Sohag Governorate in the south Egypt. This water was sent to the laboratory within 24 h and stored in the dark at 4°C until inoculation of viruses. Three flasks for each virus were used to be incubated in 4°C, 22°C and 35°C, respectively. Quantification of the number of viruses genome copies was done weekly in 50 ml of inoculated ground water for 12 weeks. Infectious units were determined from 0 time (after inoculation) and then, they were quantified weekly. Means of $\log_{10}$ reductions for the 3 inoculated viral doses (genome copies) and 3 infectious units were calculated weekly. All the samples inoculated with different viruses are from the same original sample from the same source and the same time of sampling.

Concentration of Water Samples: Water samples were concentrated by filtration through negatively charged nitrocellulose membranes (0.45 µm pore size and 47 mm diameter filter series) after addition of $AlCl_3$ to a final concentration of 0.5 mM and acidification to pH 3.5. The viruses adsorbed to the membrane were eluted with 25 ml of 0.05 M glycine buffer, pH 9.5 containing 3% beef extract (Lab-Limco powder, OXOID, UK) [18, 19]. All samples were reconcentrated using an organic flocculation method [20]. Samples were neutralized and kept at -70°C until used.

Viral Nucleic Acid Extraction: It was done Using BIOZOL Total RNA Extraction reagent (BIOFLUX, Japan) and according to the manufacturer's instructions.

Quantification of Rotaviruses in Water Samples Using Real Time RT-PCR: Real time RT-PCR was done to quantify rotavirus genome in the samples. The RT was done according to Iturriza-Gomara *et al.* [21], using primers (VP6-F and VP6-R). Real time PCR was done using power SYBR green PCR master mix (Applied Biosystem, UK) with 0.5µl of both forward primer VP6-F and reverse primer VP6-R in a total volume of 25µl. Amplification was performed as described previously [22] in a Real time PCR thermal cycler (One Step, Applied Biosystem). The specificity of the reactions was determined by melting curve analysis of the amplicons. The rotavirus genome copy number was determined by comparison with a standard curve generated with serial dilutions (10^{-1} to 10^{-6}) of a positive control plasmid (pCR2.1-TOPO, Invetrogen, USA) containing full length of cDNA of rotavirus VP-6 genome.

CC-RT-PCR for Quantification of Infectious Rotavirus Particles: It was done according to Abad *et al.* [23], El-Senousy *et al.* [24] and Ghazy *et al.* [25]. Rotavirus cell culture RT-PCR (CC-RT-PCR) assay was performed on suspensions of infected MA104 cells. Set of 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 inoculums is $1x10^1$ CC-RT-PCR units/ml (u/ml), where CC-RT-PCR u is the reciprocal end point dilution detectable by CC-RT-PCR.

Quantification of HAV in Water Samples Using Real Time RT-PCR: Real time RT-PCR was done to quantify HAV genome in the samples. The RT was done according to Sánchez *et al.* [26], using primers (HAV-68 andHAV-240). Real time PCR was done using power SYBR green PCR master mix (Applied Biosystem, UK) with 0.5 µl of both forward primer HAV-68 and reverse primer HAV-240 in a total volume of 25µl. Amplification was performed as described previously Brooks *et al.* [27], in a Real time PCR thermal cycler (OneStep, Applied Biosystem). The specificity of the reactions was determined by melting curve analysis of the amplicons. HAV genome copy number was determined by comparison with a standard curve generated with serial dilutions (10^{-1} to 10^{-6}) of a positive control plasmid (pCR2.1-TOPO, Invetrogen, USA) containing HAV HM175 cDNA.

Extraction of DNA: It was done as described previously by Kapperud *et al.* [28] and modified by Estrada *et al.* [29]. 50 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.

Quantification of Adenoviruses Using Real Time PCR: Real time PCR was done using power SYBR green PCR master mix (Applied Biosystem, UK) with 0.5 µl of both forward primer hex AA 1885and reverse primer hex AA 1913 in a total volume of 25µl. Amplification was performed as described previously [30], in a Real time PCR thermal cycler (OneStep, Applied Biosystem). The specificity of the reactions was determined by melting curve analysis of the amplicons. The adenovirus genome copy number was determined by comparison with a standard curve generated with serial dilutions of a positive control plasmid, constructed by TOPO TA cloning (Invitrogen) of the PCR product from an adenovirus positive sample.

Cell Culture-PCR (CC-PCR) Technique for Quantification of Adenovirus Infectious Units: It was done according to Esawy *et al.* [31], Abdo *et al.* [32]. Adenovirus cell culture-PCR (CC-PCR) assay was performed on suspensions of infected Hep 2 cell line. Set of primers, hex AA 1885 and hex AA 1913 was used. The detection limit in this tissue culture assay using 100 µl of inoculum is $1x10^1$ CC- PCR units/ml (u/ml), An adenovirus CC-PCR unit is defined as the reciprocal endpoint dilution detectable by CC- PCR.

Sampling of Limestone and Ground Water: The limestone was obtained from subsurface (deep about 30 meters) from Sohag area, Egypt. The samples were collected in sterile bags of plastic. In addition, ground water samples were collected in 2 liter sterile glass bottle (10 bottles) and then transferred from these sites to the Lab. in ice box after 6 hours, then, autoclaving (121°C/15 minutes) the ground water samples was done.

Preparation of the Powder from Limestone: Under sterilization, the limestone was blending and sifting, then washing by distilled sterile water (3 times). Drying the powder was done by incubation of the powder on filter paper for an hours at 37°C. Three weights were used in our experiments (1, 5 and 10 grams). All weights were put separately in sterile flasks. Each of them contained 150 ml of sterile ground water.

Composition of Limestone: Chemical composition of limestone in Sohag Governorate is shown in Tables 1 and 2 [33]. Chalky limestone in Sohag was white, snow white to whitish grey, hard and massive.

Effect of Limestone on the Persistence of Adenovirus 40, Rotavirus Wa Strain and HAV HM175 Adapted Strain in Ground Water: 1, 5 and 10 grams of natural stone from a well in Sohag Governorate were put separately in 150 ml of ground water. Different doses of adenovirus 40, rotavirus Wa strain and HAV HM175 adapted strain ($1x10^3$, $1x10^5$, $1x10^7$) genome copies were put separately in the different samples with different quantities of limestone. Continuous shaking 130 round / minute at 28°C for four days were done for all flasks. Samples from each flask were taken after 2 hours, 4 hours, 6 hours, 24 hours, 48 hours, 72 hours and 96 hours to determine the effect of lime stone on the genome copies and infectious units of tested viruses.

Cytotoxicity Test: It was done to water samples treated with lime stone with different concentrations, 1, 5 and 10 grams using cell morphology evaluation by inverted light microscopy method and cell viability assay method.

Cell Morphology Evaluation by Inverted Light Microscopy: Hep2 and MA104 cell cultures (2×10^5 cells/ml) were prepared in 96-well tissue culture plates (Greiner-Bio one). 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 tested samples prepared in DMEM (Gibco-BRL). For cell controls 100µl of DMEM without samples was added. All cultures were incubated at 37°C in a humidified 5% v/v CO_2 atmosphere for 72h. 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 [34].

Cell Viability Assay: It was done by Trypan blue dye exclusion method [35]. Hep2 and MA104 cell cultures (2×10^5 cells/ml) were grown in 12-well tissue culture plates (Greiner-Bio one). After 24 h incubation, the same assay described above for testing sample cytotoxicity was followed by applying 100 µL of tested sample dilutions

Table 1: Minerals percent in limestone of Sohag Governorate.

		Minerals percent				
Location	Sample type	Quartz	Calcite	Dolomite	Hematite	Sum
Sohag	Chalky limestone	0	96.70	0	3.3	100

Table 2: Chemical composition of limestone in Sohag Governorate

		Chemical elements per cent									
Location	Sample type	Mg	Al	Si	K	Ca	Ti	Fe	P	Na	Sum
Sohag	Chalky limestone	1	0.3	0.4	0.17	97	0.1	0.5	0.01	0.52	100

(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.

Preparation of Bacterial Isolates: From Rosetta Branch (El-Rahawy drain) of the River Nile (Egypt), water samples were collected from the subsurface layer (at depth 30 cm) in sterile bottled glass (1 liter capacity). Then, the water samples were transferred from this site to the Lab. in ice box during 2 hours. By membrane filter technique (according to APHA [36], *E. coli, Salmonella Spp. Enterococcus fecalis* and *Staphylococcus aureus* were isolated on specific media. One colony was taken from the plate which contained the specific media and put onto sterile test tube (capacity 20 ml) which contained triptcase soy broth (5 ml) and these tubes were incubated at 37°C for 24 hours. After incubation, the tubes were centrifuged at 5000 rpm for 15 minutes, then the pellets were transferred to sterile test tubes (capacity 20 ml) which contained sterile saline water (5 ml) and then vortexing was done. The final step was repeated three times.

Quantification of Bacteria Using Plate Count Agar and Membrane Filter Technique: One ml sample was taken to be counted for each bacterial type. Ten fold dilutions were done to each sample in sterile water for determination of the end dilution. In single culture flasks, plate count agar was used, while in mixed culture flask, membrane filter technique was used according to APHA [36].

Media Used for Counting Bacteria:

- Plate count agar for counting the bacteria in the single culture flasks.
- Bismuth sulphate agar for counting *Salmonella spp.* in the mixed culture flasks.
- EMB agar for counting *E. coli* in the mixed culture flasks.
- Manitol salt agar for counting *Staphylococcus aureus* in the mixed culture flasks.
- m-Enterococci agar for counting *Enterococcus fecalis* in the mixed culture flasks.

All media used were according to APHA [36].

Determination of the Persistence of *E. coli*, *Enterococcus fecalis* *Salmonella spp.* and *Staphylococcus aureus* in Ground Water at 22°C: Quantification of bacterial cfu/ml was done in 150 ml of inoculated ground water weekly for 12 weeks. *E coli, Salmonella Spp*. *Enterococcus fecalis* and *Staphylococcus aureus*, were counted using poured plate and membrane filter technique according to APHA [36] for water samples.

Effect of Limestone on the Persistence of Bacterial Indicators and Bacterial Pathogens in Ground Water: Transfer 0.2 ml from each saline water bacterial types by pipet (1 ml) onto 15 flasks, each of them contained 150 ml of sterile ground water from the same well of Sohag Governorate. The flasks were divided into three groups (5 flasks); each group contained one weight (1 g, 5 g and 10 g) of limestone. Each flask was inoculated with one bacterial isolate tested while the fifth flask contained all isolates tested. The inoculated flasks were shacked continuously at 130 round/minute at 28°C for 4 days. Samples from each flask were taken after 2 hours, 4 hours, 6 hours, 24 hours, 48 hours, 72 hours and 96 hours.

Physicochemical Analysis: In this study, the physicochemical parameters were determined in natural ground water and ground water treated with limestone according to APHA [36].

RESULTS AND DISCUSSION

Comparison Between Genome Copies and Infectious Units Reductions of the Three Tested Viruses: The decrease of the genome of adenovirus 40, rotavirus Wa strain and HAV HM175 adapted strain was observed in Figs 1, 2 and 3 which the genome of adenovirus was the more stable and persistent followed by rotavirus Wa strain and finally HAV HM 175 adapted strain. There was gradual decrease in the genome of the three tested viruses parallel with the time. After 12 weeks, adenovirus genome copies reductions were 0.05 $\log_{10}$, 0.5 $\log_{10}$ and 1.5 $\log_{10}$ at 4°C, 22°C and 35°C respectively. Rotavirus genome copies reductions were 0.1 $\log_{10}$, 0.7 $\log_{10}$ and 2.3 $\log_{10}$ at 4°C, 22°C and 35°C, respectively. HAV genome copies reductions were 0.2 $\log_{10}$, 0.8 $\log_{10}$ and 2.7 $\log_{10}$ at 4°C, 22°C and 35°C, respectively (Figs. 1, 2 and 3). Again, the decrease of the infectious units of adenovirus 40 and rotavirus Wa strain was observed in Figs 1, 2 and 3 which the infectious units of adenovirus was the more stable and persistent than rotavirus Wa strain. There was gradual decrease in the infectious units of the two tested viruses parallel with the time.

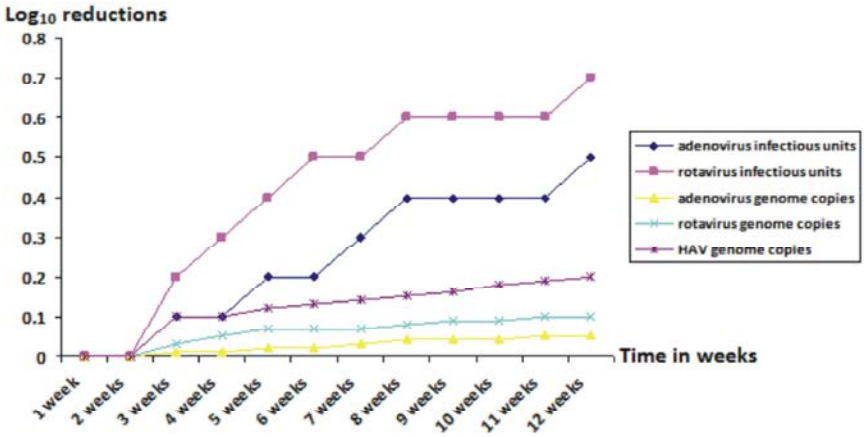

Fig. 1: Log_{10} reductions of genome copies and infectious units of adenovirus, rotavirus and HAV in ground water at 4°C.

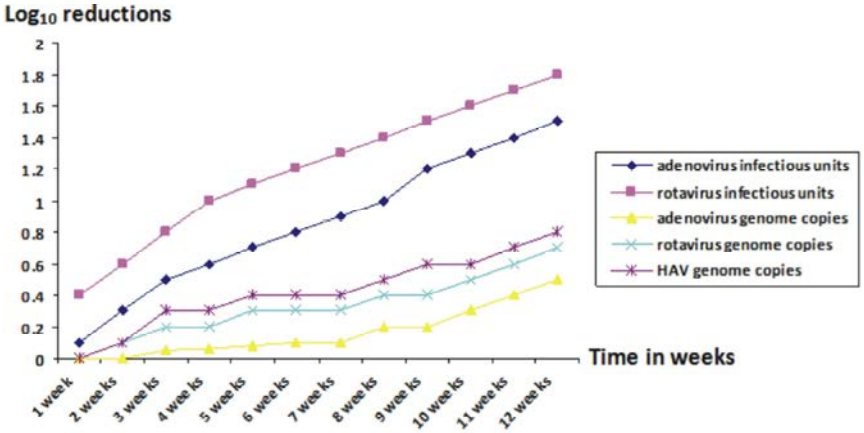

Fig. 2: Log_{10} reductions of genome copies and infectious units of adenovirus, rotavirus and HAV in ground water at 22°C.

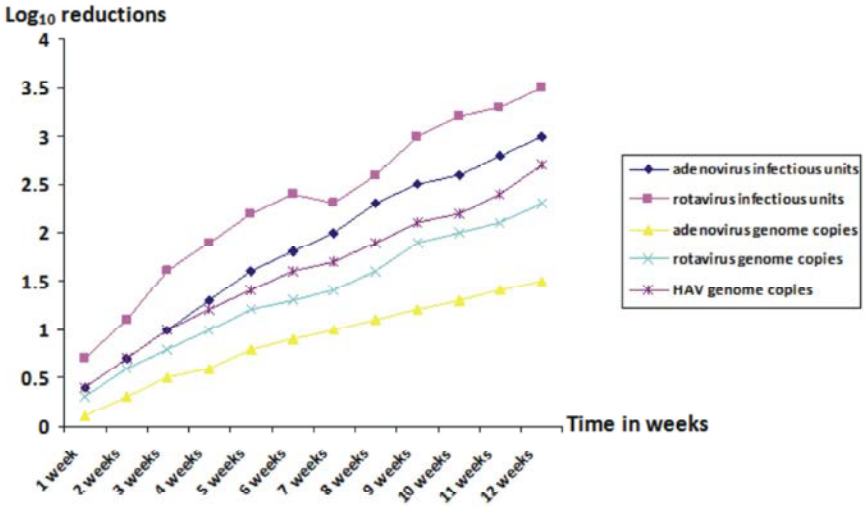

Fig. 3: Log_{10} reductions of genome copies and infectious units of adenovirus, rotavirus and HAV in ground water at 35°C.

Table 3: Physico- chemical parameters for the well water with 1g limestone

		Results		
Parameters	Unit	Well water	Well water +1g salt	Egyptian Standards 2007
pH	--	7.2	8.5	6.5-8.5
Turbidity	NTU	0.3	1	1
Odor	--	Odorless	Odorless	Odorless
Color	Co/Pt Unit	Colorless	Colorless	Colorless
Total Dissolved Solids	mg/l	239	409	1000
Total Hardness (as $CaCO_3$)	mg/l	168	168	500
Calcium Hardness (as $CaCO_3$)	mg/l	92	48	350
Magnesium Hardness (as $CaCO_3$)	mg/l	76	120	150
Calcium	mg/l	36.8	19.2	
Magnesium	mg/l	18.2	28.8	
Chloride	$mgCl_2$/l	26	160	250
Sulfate	$mgSO_4$/l	16	51.7	250
Ammonia	$mgNH_3$l	0.0	0.0	0.5
Nitrite	mg NO_2/l	0.0	0.0	0.2
Nitrate	mg NO_3/l	0.9	1.3	45
Iron	mg/l	0.06	0.4	0.3
Manganese	mg/l	0.1	0.0	0.4

After 12 weeks, adenovirus infectious units reductions were 0.5 log_{10}, 1.5 log_{10} and 3 log_{10} at 4°C, 22°C and 35°C, respectively. Rotavirus infectious units reductions were 0.7 log_{10}, 1.8 log_{10} and 3.5 log_{10}, at 4°C, 22°C and 35°C respectively (Figs 1, 2 and 3).

The genome of adenovirus 40 was the most stable among the three tested viruses. Also, it was more stable than rotavirus Wa strain in infectivity. In the study of Ogorzaly *et al.* [37], the reduction of infectivity within 120 days reached only 2.4 and 0.7 log_{10} at 20°C and 4°C, respectively for human adenovirus 2. The reported differences in the virus survival times may be due to several factors, including the type and the structure of virus, the viral strain employed and the external conditions of the experiment, as well as the aggregation status of the virus and the adsorption of the virus to particulate material or the inner surface of the container. The characteristics of the well water may also affect the survival period. The characteristics of well water used in our experiment are shown in Table 3. Enriquez *et al.* [38] for infectious adenovirus serotype 41; they suggested that adenoviruses have significant survival rates in groundwater. In contrast, Charles *et al.* [39] reported a rapid decrease in the infectivity of HadV2 at 12°C, with a reduction of 4.2 log_{10} over the initial 21 days of the study. Nevertheless, these authors also observed that infectious HadV2 could still be detected over a 364-day period. The longer persistence of the adenovirus genetic marker, compared to that of rotavirus Wa strain and HAV HM175 adapted strain, could be, at least in part, related to the nature of DNA/RNA molecules, since double stranded DNA genome (such as that of adenovirus) is known to be generally more stable than double stranded RNA genome (such as that of rotavirus) and also single stranded RNA genome (such as that of HAV).

The reduction of infectivity was faster than the reduction of genetic markers at 4°C, 22°C and 35°C for adenovirus 40 and rotavirus Wa strain. This comparison of infectivity and genetic markers is consistent with previous studies performed with poliovirus, coxsackievirus and feline calicivirus seeded in mineral water [40] or artificial groundwater [41] and naturally contaminated well water samples in El-Sadat city, Egypt [1]. As expected, a strong effect of the temperature on viral stabilities was observed. The effect of temperature in the long persistence of either genome or infectious units of enteric viruses is very clear in our results. Very low reductions were observed either for genome of the tested viruses or infectious units of adenovirus and rotavirus at 4°C even after 12 weeks. Lower persistence was observed with the elevation of temperature to 22°C and 35°C for either genome copies or infectious units of the tested viruses (Figs 1, 2 and 3).

Higher stability for the genomes of the three tested viruses and the infectious units of adenovirus and rotavirus than *E coli*, *Enterococcus fecalis Salmonella spp.* and *Staphylococcus aureus* in ground water at 22°C after 12 weeks was observed (Figs 1, 2, 3, 4 and 5).

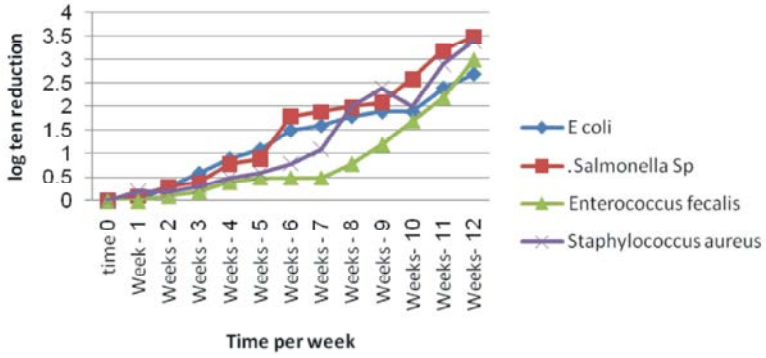

Fig. 4: Log_{10} reductions of bacterial forming units of *E coli*, *Enterococcus fecalis Salmonella sp*. and *Staphylococcus aureus* which seeded in mix in ground water at 22°C.

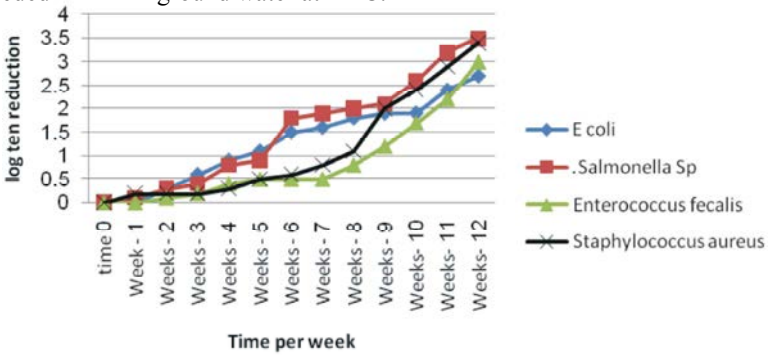

Fig. 5: Log_{10} reductions of bacterial forming units of *E. coli*, *Enterococcus fecalis Salmonella sp*. and *Staphylococcus aureus* which seeded separately in ground water at 22°C.

On the other hand bacterial indicators (*E coli* and *Enterococcus fecalis*) had higher stability than pathogenic bacteria *Salmonella Sp* and *Staphylococcus aureus* (Figs 4 and 5). The long survival of adenoviruses genome till 1 year was recorded previously [37]. They showed higher stability of adenovirus type 2 than MS2 phages and GA phages. On the other hand, infectious enteric viruses commonly persist longer in the environment than do fecal bacteria [9]. Also, the viral genome has a longer persistence than does an infectious particle [10].

Survival of Tested Bacterial Strains in Ground Water: The initial count for all isolates was around 10^4 CFU/ml. Fig. 4 showed that, the tested strains could be detected after 12 weeks in both single and mixed cultures. Log_{10} reductions of bacterial strains in mixed culture were higher than in single culture, where 2 log_{10} reduction were recorded after 9 weeks for *Salmonella spp.* and *Staphylococcus aureus,* respectively. The same reductions needed 11 weeks for both *E. coli* and *Enterococcus fecalis*. This may be due to the competition between the bacteria on different conditions like pH or nutrient on the media. In addition, one log_{10} reduction was recorded for gram positive bacteria after 5 weeks and for gram negative bacteria after 7 to 9 weeks.

The long survival of bacterial indicators agrees with the data of Fusconi and Godinho [42], when counted the total bacteria in ground water in Brazil for 12 months. As well as they concluded that bacteria may use different organic pollutants as substrate for regrowth. Also, Anderson *et al.* [43] noticed that fecal coliforms and enterococci indicators had more survival than other bacteria tested for one month in sediment tank during water treatment. On the other hand, our results agree with APHA, [36] and EPA [44] which recommended that, bacterial indicators can survive more than the other bacteria in different environments. Grisey *et al.* [14] noticed that, total coliforms, *Escherichia coli, Enterococci*, *Pseudomonas aeruginosa, Salmonella sp*. and *Staphylococcus aureus* could be detected for 15 months in groundwater.

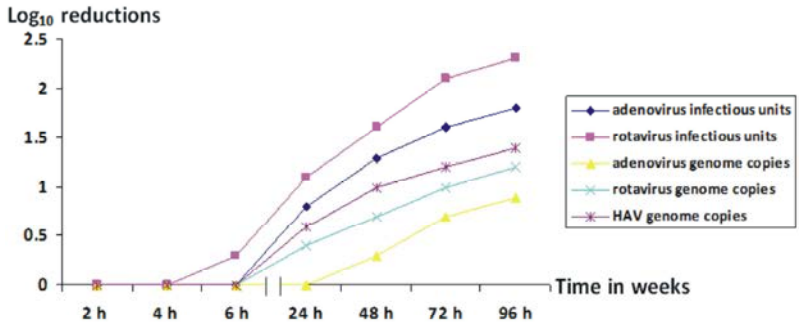

Fig. 6: Effect of limestone (1 g) on the persistence of genome copies and infectious units of adenovirus 40, rotavirus Wa strain and HAV HM 175 adapted strain in ground water.

Effect of Limestone on the Persistence of Adenovirus 40, Rotavirus Wa Strain and HAV HM 175 Adapted Strain in Ground Water: The decrease of the genome of adenovirus 40, rotavirus Wa strain and HAV HM 175 adapted strain as a result of water treatment with 1g limestone was observed in Fig. 6. The highest stability was observed for adenovirus 40 genome followed by rotavirus Wa strain genome and then HAV HM 175 adapted strain genome. Higher stability of adenovirus type 40 and rotavirus Wa strain genomes than the infectious units of both viruses was observed. Also, higher stability of adenovirus infectious units than rotavirus infectious units was observed (Fig. 6). Until 6 hours there was no effect on genomes of tested viruses and infectious units of adenovirus 40. The only effect after 6 hours was on the infectious units of rotavirus Wa strain 0.3 $\log_{10}$ reduction. Considerable effect on either viral genome or infectious units was observed after 24 hours Fig. 6. The effect increased gradually with time. After 96 hours, the mean reductions of genome copies and infectious units were 0.9 $\log_{10}$, 1.2 $\log_{10}$ and 1.4 $\log_{10}$ for adenovirus type 40, rotavirus Wa strain and HAV HM 175 adapted strain genome copies respectively and 1.8 $\log_{10}$ and 2.3 $\log_{10}$ for adenovirus type 40 and rotavirus Wa strain infectious units respectively in ground water treated with limestone 6.7 g/l. The physicochemical parameters for water treated with 1 gm lime stone were in the available limits according to the Egyptian drinking water quality standard Table 3. In case of water treatment with 5 g and 10 g limestone, the physicochemical parameters for water were higher than the available limits according to the Egyptian drinking water quality standard. The cytotoxicity test indicated higher toxicity in water treated with 10 g limestone followed by 5 g. There was no cytotoxicity in case of water treated with 1gm limestone. The effect of treatment of water with limestone on tested viruses depends on the high pH caused by the lime which was 8.5 in case of adding 1 g limestone. The effect of limestone on either bacterial indicators or pathogenic bacteria was faster and higher than its effect on viral genome and infectious units (Figs 7 and 8). Again, bacterial indicators had higher persistence than pathogenic bacteria (Figs 7 and 8).

Effect of Limestone on the Tested Bacteria: Fig. 7 shows that, after 2 and 4 hours, 1 $\log_{10}$ reduction was recorded for *E coli* and *Salmonella spp*. respectively in the single culture when mixed with one gram limestone tested (calcium carbonate about 97%), while this one $\log_{10}$ reduction was recorded after 48 hours for *Enterococcus fecalis* and *Staphylococcus aureus*. With regard to Fig 8, in mixed cultures, the same values were recorded after 24 hours for *E. coli* and *Salmonella Spp*. and after 48 hours for *Enterococcus fecalis* and *Staphylococcus aureus*. This result is in agreement with the data of EPA [44] which reported that limestone (karst) and other stones were barriers for faecal contamination, chemical and physical pollutants. Our results disagree with David, 2003 in USA, who found that from the initial count (10^2), one $\log_{10}$/ml decrease was realized after 4 days when ground water saturated with limestone was tested at 22°C. Ataee *et al.* [45] recommended that, calcium carbonate is a good antibacterial material and it can be used in different fields of industry, agriculture and can be of importance considering health as well as economic issues. Furthermore, our results showed that the physicochemical (pH, TDS, BOD and TSS….etc) were in the available limits according to the Egyptian drinking water quality standard. Also, no cytotoxicity was observed. Also, *Enterococcus fecalis* and *E. coli* had more survival rate than other tested bacteria in both single and mixed cultures. Also bacteria in mixed cultures had more survival rate than single cultures in the presence of limestone. These results are in agreement with those obtained by Sepehrnia *et al.* [11] and Mara and Johnson [17].

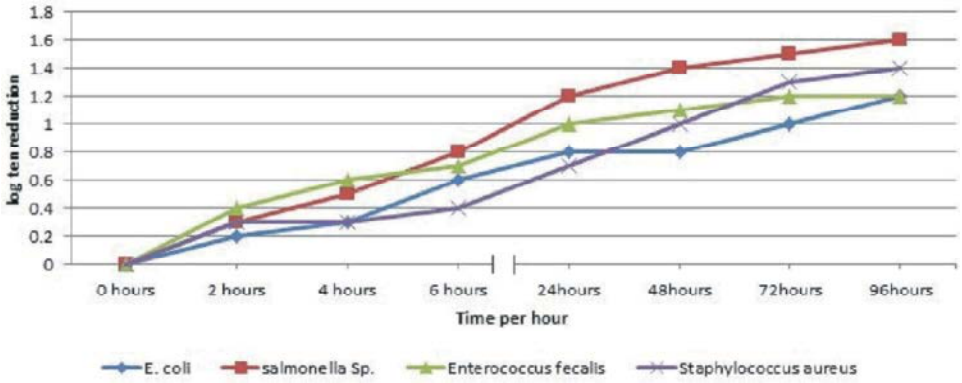

Fig. 7: Effect of limestone (1 g) on the persistence of *E coli*, *Enterococcus fecalis*, *Salmonella sp*. and *Staphylococcus aureus* which seeded separately in ground water.

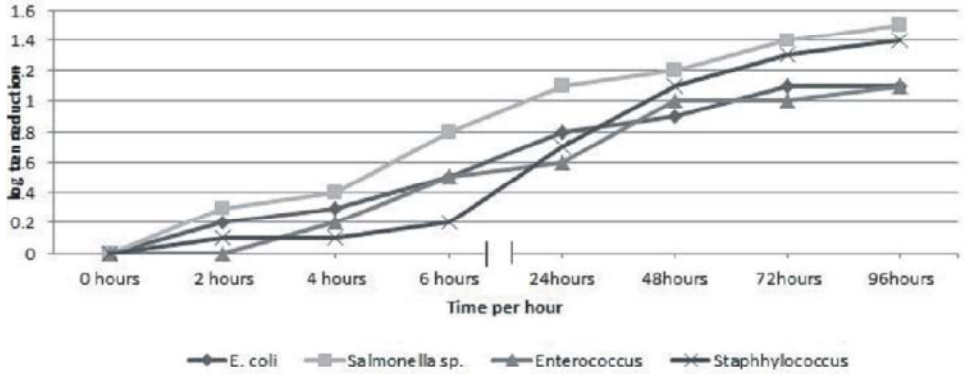

Fig. 8: Effect of limestone (1 g) on the persistence of *E. coli*, *Enterococcus fecalis*, *Salmonella sp*. and *Staphylococcus aureus* which seeded in mix in ground water.

CONCLUSION

It could be concluded that the genome of adenovirus 40 is more persistent than the genome of rotavirus Wa strain and HAV HM 175 adapted strain in ground water at 4°C, 22°C and 35°C for 12 weeks and at higher pH caused by limestone. Also, the adenovirus 40 infectious units are more persistent than the infectious units of rotavirus Wa strain at the same temperatures and pH. The genome and infectious units of all tested viruses are more persistent than either bacterial indicators or bacterial pathogens. At the same time, bacterial indicators are more persistent than bacterial pathogens. On the other hand, the effect of limestone on either bacterial indicators or pathogenic bacteria was faster and higher than its effect on viral genome and infectious units.

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