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Novel "Superspreader" Coliphages for Detecting Microbial Water pollution

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ABSTRACT

Water is one of the most important bacterial habitats on Earth. The present study focuses its aim to apply modern molecular approach for identification, characterization of novel coliphages as well as monitoring microbial water quality throughout trapping their hosts in various aquatic systems. The study was started with samples collection in two different seasons (summer and winter, 2017-2018). The area covered represented two major categories of water sector in Egypt; River Nile at Rosetta branch, and drainage wastewater from five main drain outfalls. Three novel phages (C1, C2 and C3) specific to *E.coli* were isolated and characterized using *E.coli* ATCC® strain 25922 and wild type *E.coli* isolates. The three phages were reported to be highly specific to about 90% of wild type strains of *E.coli* in collected water samples. The transmission electron microscope characterized the morphological resemblance of these phages to those of the *Myoviridae, Siphoviridae* and *Podoviridae* families, while molecular analysis showed different major coat protein gene sizes (1278bp, 459bp and 774bp), respectively. The nucleotide sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database, USA and were assigned their accession numbers; MK370060.1, MK370061.1 & MK370062.1, respectively. Based on their phage specific variability, three *E.coli* isolates were chosen for molecular characterization using 16s rDNA gene sequencing. On behalf of phylogenitic tree analysis, the three Egyptian strains A1 (252bp), A2 (254bp) and A3 (217bp) showed 98% resemblance to strains from France, India, Iran and Saudi Arabia. They were assigned the accession numbers MK359106.1, KY359107.1 and MK359167.1, respectively. Using this novel coliphage isolates were able to specifically detect at least one colony forming units (CFU) of *E.coli* within 12-24 hours. The study recommended continuous monitoring and controlling various bacterial pathogens could be achieved and should be contributing to maintain of public health.

Keywords: Bioinformatics; Coliphages; Microbiological; NCBI; Water quality.

Introduction

Water is an important component of every life. Egypt is an arid country that faces challenges due to its limited water resources and disorders of water balance (Nasr and Zahran, 2015). Water quality is a description of physical, chemical and biological characteristics of water. Industrial and agricultural wastewater causes pollution of surface water (rivers and lakes) with chemicals and excess nutrients (El Gohary, 2015). Monitoring microbial water quality has been conducted for more than a century by measuring indicator bacteria that occupy human intestinal systems, primarily fecal coliforms, *Escherichia coli*, and some enterococci.

Escherichia coli is a classified as a rod-shaped, Gram-negative bacterium of many diverse types in the family Enterobacteriaceae, the majority of which are part of the normal flora of the intestine and are believed to be relatively harmless. *E.coli* include not only commensal strains but also pathogenic ones that cause a variety of human diseases resulting in more than two million deaths each year (Farrokh *et al.,* 2013). Pathogenic *E.coli* implicated in many waterborne outbreaks worldwide and represents a phenotypically and genotypically diverse group of pathogens and there is no single method to enrich and to isolate for the various pathotypes that exist (Dale and Woodford, 2015). However, some strains have evolved mechanisms of pathogenicity, meaning they can cause disease in humans and animals. Such diseases can be intestinal (diarrhoea) or extraintestinal (urinary tract

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infection (UTI), septicaemia, pneumonia and meningitis) (Chandran and Mazumder, 2015) and (Millar *et al.,* 2016). The bacterium mainly inhabits the lower intestinal tract of warm-blooded animals, including humans, and is often discharged into the environment through faeces or wastewater effluent (Balière *et al.,* 2015).

Aquatic bacterial pathogens pose a global health threat and cause extensive morbidity and mortality each year. Bacteriophage-based assays are solved and designed to detect a specific host, however, diagnostic assays capable of detecting and discerning between wide ranges of pathogens simultaneously is of great environmental utility. Bacteriophages offer unique features which could be utilized to create novel, cheap and effective diagnostics for aquatic bacterial pathogens (Wu *et al.,* 2017). Now, many isolated phages have been used for controlling of drainage and surface water bacterial pathogens such as *S.typhi, P.vularis, E.faecalis* and *E.coli* (Periasamy and Sundaram, 2013).

Escherichia coli phages (coliphages) are commonly isolated from different aquatic environments and sources include: sewage, hospital waste water, polluted rivers and fecal samples of humans or animals (Song *et al.,* 2007; Clokie and Kropinski, 2009 and Dalmasso *et al.,* 2016). The most well-known phage-based methods employed for detection of some important bacteria include: plaque assay, phage amplification, reporter phage, phage-labeling and phage capture elements (Funatsu *et al.,* 2002; Griffiths, 2010 and Smart and Ripp, 2011).

The present research has been done with the objective of developing and applying of a novel characterized coliphages for rapid detecting *E.coli* in water. Behind monitoring of microbial water quality, being a model index for water born opportunistic pathogen of public health concern. The modern biotechnological analyses were applied to verify the detected bacteria and their phages.

Materials and Methods

Study area

The area in our study was chosen to represent two major water sectors in Egypt; River Nile and drainage water. It extended about 120 Km in the River Nile at Rosetta branch. The branch was subdivided into five reaches based on locations of known waste inputs as illustrated in Table 1. Totally fifteen sites were chosen, three from each reach: five at drain outfalls (El-Rahway, Sabal, El-Tahreer, Zawiet El-Bahr and Tala) and ten sites in Rosetta branch (five upstream and five downstream those drains outfalls).These are mixed drains from sewage, agricultural and industrial wastes.

Table 1: Location of the study sites in Rosetta branch and drains.

Site code	Description	Latitude (N)	Longitude (E)
R1	Rosetta branch, upstream El-Rahawy drain	30°12'30.23"N	31°2'02.21"E
R	El-Rahawy drain outlet (left bank)	30°12'26.21"N	31°1'58.90"E
R2	Rosetta branch, downstream El-Rahawy drain	30°12'25.41"N	31°1'48.35"E
S1	Rosetta branch, upstream Sabal drain	30°31'57.94"N	30°50'53.20"E
S	Sabal drain outlet (right bank)	30°32'13.47"N	30°51'07.09"E
S2	Rosetta branch, downstream Sabal drain	30°32'29.05"N	30°51'01.27"E
G1	Rosetta branch, upstream El-Tahreer drain	30°36'23.50"N	30°47'51.89"E
G	El-Tahreer drain outlet (left bank)	30°36'24.68"N	30°47'48.92"E
G2	Rosetta branch, downstream El-Tahreer drain	30°36'28.90"N	30°47'47.49"E
Z1	Rosetta branch, upstream Zawiet El-Bahr drain	30°42'51.10"N	30°45'55.23"E
Z	Zawiet El-Bahr drain outlet (left bank)	30°42'52.57"N	30°45'19.01"E
Z2	Rosetta branch, downstream Zawiet El-Bahr drain	30°43'09.26"N	30°45'39.53"E
T1	Rosetta branch, upstream Tala drain	30°48'58.19"N	30°48'37.60"E
T	Tala drain outlet (right bank)	30°49'01.74"N	30°48'47.77"E
T2	Rosetta branch, downstream Tala drain	30°49'10.36"N	30°48'42.11"E

Collection of water samples

The study was started with samples collection in which 60 water samples were collected in duplicates, 40 from Rosetta branch and 20 from drains outfalls as described above. Samples collection was processed in two different seasons (summer & winter, 2017-2018) according to Standard Methods for Examination of Water and Wastewater (APHA, 2012). All collected samples were stored in an iced cooler box and delivered to CLEQM-NWRC where it has been analyzed.

Quantitative assay of *E.coli* in water samples

Classical methods using membrane filter assay as well as non-conventional methods using phage-based diagnostic, phylogenetic and molecular analysis were applied simultaneously as follows:

Membrane filter technique

Standard method No. 9213 E (APHA, 2012) was followed. Water samples were filtered through sterile, white, grid-marked, 47 mm diameter membrane with pore size 0.45µm which retained bacteria. After filtration, the membrane was plated on m-TEC agar medium (Difco, USA) and incubated at 35ºC/2hr then 41.5ºC/24h. Results were recorded as colony forming unit (CFU 100 ml^{-1}) using the following equation:

$$Colonies\ /100\ ml = \frac{Counted\ colonies}{ml\ of\ sample\ filtered}\ X\ 100$$

These colonies were confirmed by streaking on EMB agar (Difco, USA) plates, a selective medium which inhibits bacterial growth except *E.coli* and enhances greenish metallic sheen colonies. Confirmation and verification was completed by microscopical and biochemical examinations (Gram staining, pigment production and oxidase test) according to Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005) as well as by Analytical Profile Index (API 20 NE) assay (bioMérieux, France) according to Juang and Morgan (2001). The whole assay lasts for 3-4 days for completion.

Qualitative and quantitative assay of phages

Water samples (100ml) were filtered through 0.45-µm pore size polycarbonate membranes (Millipore, Bedford, MA, USA). The filtrate was added to a fresh bacterial culture of *E.coli* (ATCC® strain 25922) in tryptic soy broth (TSB) (Difco, USA) and incubated at 37°C for 5 h at 100 rpm, then centrifuged (10000 × *g,* 10 min) (ThermoHeraeus Pico, Hanau, Germany) and filtered through a 0.2µm membrane (Millipore). Phage isolation was done by the double-layer method using tryptic soy agar (TSA) (Difco, USA). The plates were incubated at 37°C and examined for the presence of plaques after 12h. The plaque forming units were calculated according to Stephenson (2010) using the following equation:

$$Plaque\ forming\ units\ (PFU)/ml = \frac{(Number\ of\ plaques)\ x\ (Dilution\ Factor)}{Phage\ volume\ plated\ (ml)}$$

Two more successive single-plaque isolations were performed to obtain a pure phage stock. All lysates were filter-sterilized. The plaque diameter was determined in double-layer cultures. Phage stocks were stored at 4°C with 1% chloroform (Clokie and Kropinski 2009; Mohan Raj *et al.,* 2018).

Host range test

The host range of the phage lysate was done by spot tests against *E.coli* (ATCC® strain 25922) and *B.subtilis* (ATCC® strain 23857) on TSA. For each bacteria, 10µl of high titer phage stock (10^{10} $PFUmL^{-1}$) was spotted on the surface of a plate cultured by the tested strain. Each test was done in duplicate.

Transmission electron microscopy (TEM)

Purified phages were dropped onto a carbon-coated copper grid for the adsorption of the phages. After 3 min, the phage-loaded grid was left to dry on a filter paper, then negatively stained by using 4% phosphotungestic acid or 2% uranyl acetate for 2 min, and left to dry for 15 min and finally examined by energy-filtering transmission electron microscopy (EF-TEM; JEM1010, JEOL, Tokyo, Japan) (Electron Microscope Unit, Assuit University). Phages were classified morphologically according to the guidelines of the International Committee on Taxonomy of Viruses (ICTV).

Viral DNA sequencing and bioinformatic analyses

Total DNA of the three purified phages were extracted using DNeasy plant minikit (QIAGEN) protocol was used as directed by the manufacturer instructions, and the genomic DNA was visualized on 0.8% (w/v) agarose gel. The primers specific for coliphages were designed according to major coat protein (MCP) gene sequences recorded and available in GenBank. The specific primers were selected from six primer sets based on temperature annealing (Tm) and % GC as shown in Table 2.

Table 2: Primer design specific for major coat protein gene sequences of isolated coliphages.

Primers	Sequence	Tm °C	% GC	Expected size
Forward	5' CTGGTCGTGTTCAGCAGACT 3'	60.47	50.00	1278bp
Reverse	5' AGCCATAAGAGCAGGATCGC 3'	60.18	55.00	
Forward	5' GCGTGATGGTTGGGATGGTA 3'	60.21	60.00	459bp
Reverse	5' GACGCTCAATCTGACGACCA 3'	50.00	55.00	
Forward	5' CCGCGATTGCGAGCATTAAA 3'	60.67	50.00	774bp
Reverse	5' CGGTCTGAATGTTCACCGGA 3'	60.53	55.00	

PCR reaction was performed in 30µl volume tubes according to Williams *et al.,* (1990) that contained the following: $dNTP_s$ (2.5 mM), 3.00µl; $MgCl_2$ (25mM), 3.00µl; PCR-buffer (10X), 3.00µl; Primer (10 P mol), 2.00µl; Taq DNA polymerase (5u/ml), 0.20µl; Template DNA (25ng), 2.00µl; $d.H_20$, 16.80µl using an automated thermal cycle (model techno 51z) programmed as follows:1 min at 94ºC, 1 min at 60ºC, and 2 min at 72ºC. The reaction was finally stored at 72ºC for 10min.

Sequencing for isolated phages were carried out in MC Lab., USA (www.mclab.com), using the dideoxyribonucleoside chain termination procedure originally developed by Sanger *et al.,* (1977). The nucleotide sequences were determined automatically by the electrophoresis of the cycle sequencing reaction product on 310 Genetic Analyzer. The data were provided as fluorimetric scans from which the sequence was assembled using the sequence analysis software.

The nucleotide sequences of DNA were identified using the Basic Local Alignment Search Tool (blast) on the NCBI database (http://www.ncbi.nlm.nih.gov). The identified nucleic acid sequences were then translated to the corresponding peptide sequences using Transeq EMBOSS programs (The European Molecular Biology Open Software Suite) on (http://www.ebi.ac.uk/Tools/st).

Multiple alignments of sequences were performed using DNAMAN software (Madison, Wisconsin, USA, version 5.2.9) and Clustalw program (version 1.74) (Thompson *et al.,* 1994). The nucleotide distances were estimated considering alignment gaps and using the juckes and cantor's method (Jukes and Cantor, 1969) for correction of superimposed substitutions with the Molecular Evolutionary Genetic Analysis (MEGA) software (version 7.0) (Tamura *et al.*, 2013).

Phylogenetic relationships among identified coliphages were evaluated using unweighting pair Group Method with Arithmetic Mean (UPGMA) through DNAMAN software and Neighbor Joining (NJ) implemented through MEGA (version 7.0) software, and boot strap analysis (1000 replicates) was performed to assess the reliability of the constructed phylogenetic tree. The nucleotide sequences data determined here were finally submitted in the National Center for Biotechnology Information (NCBI) GenBank database, USA, and assigned their accession numbers.

Bacterial DNA sequencing and phylogenetic analyses

Genomic DNA of *E.coli* strains were extracted using Bacterial Genomic DNA Isolation Kit RKT09 (Chromous Biotech Pvt. Ltd., Bangalore, India) and visualized on 0.8% (w/v) agarose gel. Gene amplification was carried out by using a Thermal cycler (ABI 2720) in 100µl reaction volume containing 2.5mM of dNTP, 10x PCR buffer, 3U of Taq DNA polymerase, 10ng template DNA, and 400ng of primer (F) 5'-GTGTCCATTTATACGGACATCCATG-3', and primer (R) 5'-CCTATAACGTCATGCCAATATTGCC-3' which were designed for *E.coli* according to Miyazaki *et al.,* (2017). The amplification program was set as an initial denaturation at 94°C for 5min., followed by 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 2min and a final extension at 72°C for 5min. The sequencing reactions were performed using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit and analyzed on an ABI Prism 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The sequences were compared with recorded 16S rDNA gene sequences in the EMBL database using a BLAST server at the European Bio-Informatics Institute (EBI; http://www.ebi.ac.uk, Hinxton Hall, Cambridge, UK). DNA sequences were aligned using CLUSTAL W (Thompson *et al.,* 1994). Phylogenetic analyses were performed using the neighbour-joining (NJ) method to test the support for the phylogeny with a bootstrap analysis based on 1000 replicates using MEGA ver. 7.0 (Tamoura *et al.,* 2013).

Prediction of protein structure

The protein structure homology modeling for the phages were designed and built in three-dimensional protein structure model using the Swiss Model software on the NCBI database (http://swissmodel.expasy.org).

Statistical analysis

The data was analyzed using SPSS software version 12.0.0.1 to calculate Min, Max, and Mean values of measured parameters. As well as MS EXCEL software version 2010 was used to calculate percentages, log transformed data and Pearson's correlation coefficient (r).

Results and Discussions

Quantitative assay of *E.coli*

Our results using membrane filter system showed that *E.coli* was detected in all examined water samples. Levels of prevalence were evaluated through the recorded counts in cfu100ml^{-1}. For better data illustration, the counts were expressed as 10 log cfu 100ml^{-1} and demonstrated as given in Figure 1.

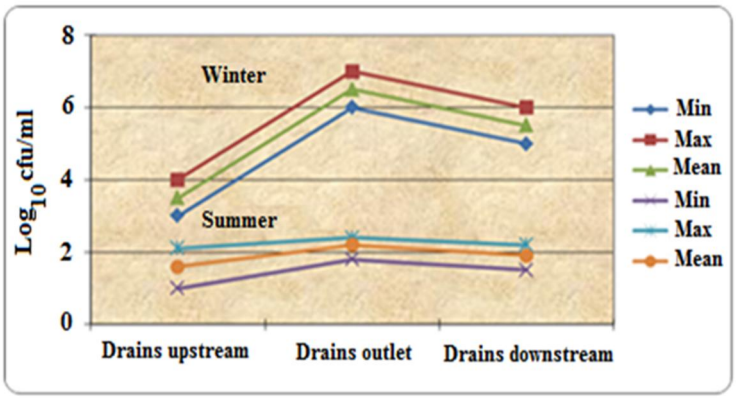

Fig. 1: the log $_{10}$ of total count of *E.coli* detected in water samples.

The log $_{10}$ of detected counts revealed gradual increase from upstream to downstream in Rosetta branch, presumably due to direct discharge from drains. Not surprisingly, maximum mean counts were recorded at drains outfalls ranging between 220-160 cfu100ml^{-1} in summer and winter seasons, respectively. Meanwhile, *E.coli* counts in Rosetta branch downstream the drains were superior to those at upstream locations. Mean counts fluctuated between 90-110 cfu100ml^{-1} at downstream and 80-100 cfu100ml^{-1} at upstream, respectively in summer and winter seasons.

Earlier investigators detected the highest pollution levels in Rosetta branch downstream drains outfalls, and addressed the problem to the combined influence of industrial, agricultural and domestic wastes discharge (Donia 2005; Ezzat *et al.,* 2012 and Azzam *et al.,* 2017). In this respect, Health Canada has established guidelines for *E.coli* in water of ≤200 cfu100ml^{-1} in case of primary human body contact, and ≤400 cfu100ml^{-1} for secondary body contact (Health Canada, 2010).

Although it is expected that, temperature increase in summer could be ideal for prolonged survival and multiplication of bacteria in water, results demonstrated that, highest levels of *E.coli* were recorded during winter rather than summer season. This phenomenon could be attributed to accumulation of wastes in drains which is usually accomplished by winter closure. In most cases,

increasing discharge at high flood period improves water quality in summer months (Yousry *et al.,* 2009).

As shown previously, the spatial distribution of *E.coli* within different water resources particularly moderate and highly polluted areas indicates its ubiquitous nature in aquatic environment and its importance as water born opportunistic pathogen of growing concern to public sector. The same observations were also reported by Sidhu *et al.,* (2013) and Um *et al.,* (2016).

Prevalence and distribution of *E.coli* in water

Out of 60 water samples processed in duplicates during two different seasons, 180 presumptive *E.coli* colonies were isolated from membrane filter assay. 90 (50%) from drains outlet, 70 (39%) and 20 (11%) respectively from drains downstream and upstream in Rosetta branch (Figure 2).

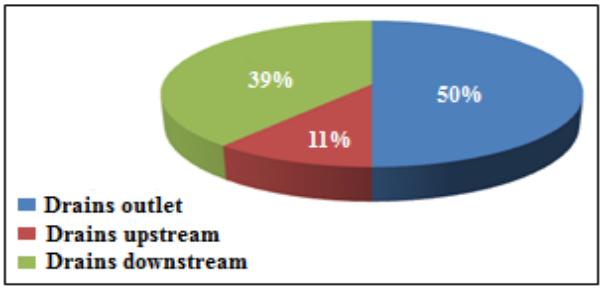

Fig. 2: Percentage of *E.coli* colonies in different locations.

These colonies were typically 0.8 to 2.2mm in diameter and flat in appearance with grayish, white, moist, smooth and opaque. Streaked colonies on MacConkey agar showed enhanced flat and pink color, while microscopic examination revealed Gram-negative, non-spore forming bacilli. Phenotypic identification and verification were further processed using API 20NE assay.

In our study, only 135 isolates representing about 75% of total isolates were confirmed as typical *E.coli*, meanwhile 45 (25%) were classified as being atypical colonies not belonging to *E.coli*. the Pearson's correlation coefficient (r) used to correlate true and false positive results of detected bacteria indicated highly significant difference ($P < 0.01$) at 0.99995 (Table 3). Indeed it seems possible to address that, data misidentification reported through this study and constituted about 25% is considered as non-ignorable bias, particularly when we are dealing with bacteria associated with human infections (Gould *et al.,* 2012). Our results also matched those recorded by Olowe *et al.,* (2017) who discovered 30% misidentification using classical methods.

Table 3: Total Number and percentage of *E.coli* strains detected in different regions.

Sites	*E.coli* colonies			
	Identified No.	Percentage	Non-Identified	Percentage
Drains outlet	70	77.8	20	22.2
Drins upstrem	15	75	5	25
Drains downstream	50	71.4	20	28.6
Total	135	75	45	25

The identification by traditional techniques could not overwhelm the problems of marked phenotypic variability demonstrated by *E.coli* and other closely related species (Joyanes *et al.,* 2001 and Jang *et al.,* 2017). Also, they usually fail to detect bacteria that become non culturable due to environmental stress. Lag time needed (2-3 days) for test completion could certainly postpone identification of contamination source and implementation of effective control measures (Bergeron *et al.,* 2011 and Ettenauer *et al.,* 2015).

Qualitative and quantitative assay of coliphages

Bacteriophages are viruses which have the ability to infect bacteria and multiply only within their cells, hence they are detectable wherever their specific host (bacteria) exist (Maheux *et al.,* 2011; George *et al.,* 2013 and Li *et al.,* 2017). In our study, the American Type Culture Collection *E.coli* (ATCC® strain 25922) was employed as a reference host to detected of phages in water by spot test.

Clearly, Figure 3 demonstrates the incidence of *E.coli* phages in all tested water samples (n= 60), being maximum (10^9-10^{10} pfu ml^{-1}) at drains outlet and minimum (10^3 pfu ml^{-1}) at drains upstream in Rosetta branch. Concentrations were obviously higher in winter than in summer, most probably due to sunlight effect which is known to be a pertinent factor governing the incidence of viruses and phages in the environment (Kohn *et al.,* 2016 and Chen *et al.,* 2017).

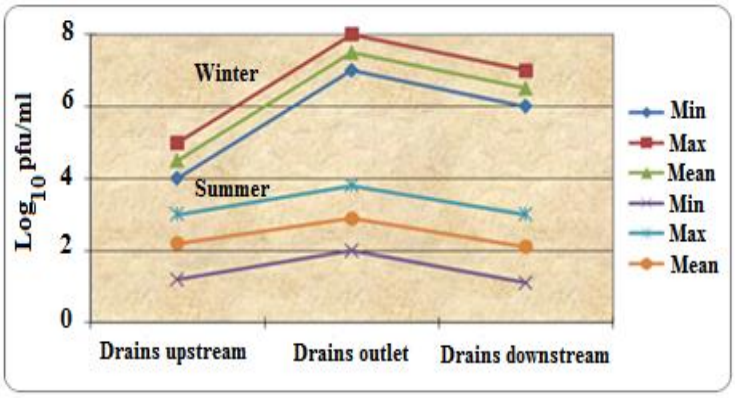

Fig. 3: The log $_{10}$ of total count of coliphages detected in water samples.

Our results confirmed that, number and behavior of phages were directly influenced by the densities of their specific host. As well as, phages have a high probability of encountering their host bacteria due to continual movement in water (Yahya *et al.,* 2015). Many studies discussed and applied the detection of *E.coli* phages, particularly from sewage water and aquatic ecosystems subjected to sources of microbial pollution (Jamal *et al.,* 2015; Dallall *et al.,* 2016; Amarillas *et al.,* 2017 and Liu *et al.,* 2018).

The plaques characteristics were determined after plaque assay technique using different bacterial strains of *E.coli* and appeared clear zones (Figure 4). The plaques size ranged between 2-5mm in diameter, which were circular and regular in morphology. Similarly, both of Valério *et al.,* (2017) and Litt *et al.,* (2018) detected and characterized *E.coli* phages of nearly comparable plaque sizes (2-4mm diameter) from drainage water and Ganges river water, respectively. In our study noted that, dramatic decrease in *E.coli* cells on behalf of phage infection during reduction assay. The $O.D_{600}$ of bacterial titer (0.815nm) dropped to 0.238nm upon mixing with phage after 24h of incubation.

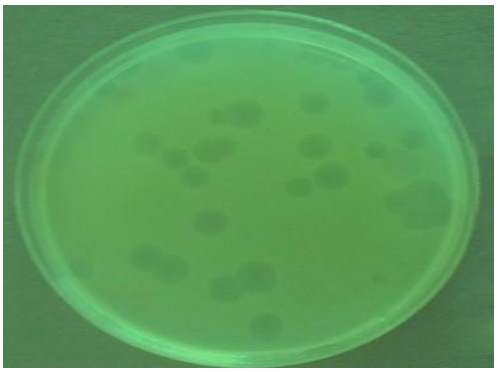

Fig. 4: Plaque assay of *E.coli* phages after incubation at 37°C/24h.

Host range pattern

The plaque assay of newly isolated coliphages is largely dependent on the ability of the phage to specifically target its host species, and to infect as many strains as possible. The presence of plaques "host clearing" is being indicative of host-susceptibility (Schofield *et al.,* 2012). In our study, an attempt has been made to evaluate the specificity and host range pattern of three isolated phage stock (C1, C2 & C3) and ensure the ability of these wild-type phages to target as many *E.coli* strains as possible in water samples.

Table 4: Host range pattern of newly phages against different *E.coli* strains**.**

Sites	Bacterial hosts	No. of used strains	No. of used strains	Percentage
Drains outlet	*E. coli*	70	66	94.3
Drins upstrem	*E. coli*	15	10	66.7
Drains downstream	*E. coli*	50	43	86.0
Reference strains	* *E. coli* ATCC® strain 25922	1	1	100
	* *B. subtilis* ATCC® strain 23857	1	0	0

* American Type Culture Collection (reference strains).

Among 180 typical *E.coli* isolates from various water sources the coliphage stock was highly specific to 90% of tested strains compared to ATCC® strain 25922 as a positive control. Phage specificity reached about 94.3% in highly polluted samples (Drains outlet), followed by Rosetta branch after drains discharge (86.0%) and before discharge (66.7%) as shown in Table 4. Normally, the susceptibility to phage infection was expected to be more pronounced in highly polluted sites, most likely due to direct relationship between phage and host densities (Yahya *et al.,* 2015).

On the other hand, data misleading didn't exceed 14.0% compared to those obtained by membrane filter assay (39.2%), and the time elapsed for test completion didn't exceed 24h to get results. Additionally, the use of multiple lytic phages in the same sample ensured higher specificity. Same conclusions were reported by Edgar *et al.,* (2006) and Maheux *et al.,* (2011).

Transmission electron microscopy (TEM)

TEM imaging of partial purified phage particles revealed different structural characteristics and dimensions as demonstrated in Table 5 and Figure 5.

Table 5: Morphological characterizations of environmental lytic coliphages as determined by TEM.

Phage Isolates	Families	Head capsid (nm)		Tail (nm)	
		Length	Diameter	Length	Diameter
C1	*Myoviridae*	86±4	86±4	180±7	14±0
C2	*Siphoviridae*	65±3	65±3	206±10	20±7
C3	*Podoviridae*	87±7	80±7	47±3	26±0

The three phages are non-enveloped and have icosahedral capsid. C1 & C2 have long non-contractile tail, while C3 has short non-contractile tail. According to International Committee on Taxonomy of Viruses (ICTV), the isolated phages belonged to the *Myoviridae*, *Siphoviridae* and *Podoviridae* families. These two families have been documented to include many phages which have the ability to infect members of Enterobacteriaceae (Jamalludeen *et al.,* 2008; Lavigne *et al.,* 2009 and Luhtanen *et al.,* 2014).

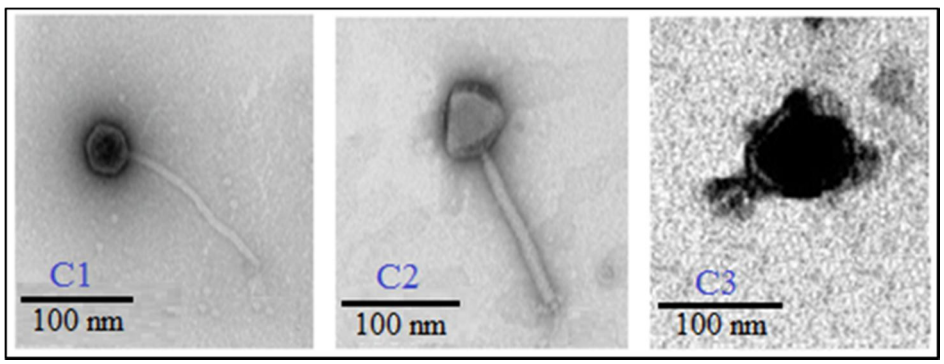

Fig. 5: Electron micrographs of novel Egyptian coliphage particles.

Molecular identification and characterization of phages

Sequencing of DNA for the three coliphages (C1, C2& C3) showed different cp-gene sizes (1278bp, 459bp &774bp), respectively. All nucleotide sequences were aligned with other major coat protein sequences of coliphages published in the international databases (NCBI, EMBL and DDBJ). The Multiple sequence alignment revealed diversity among novel Egyptian coliphage isolates (C1, C2, C3) compared with other isolates recorded in GenBank including both of: Phage M, Phage D11, Phage 74 (USA), Phage D (Canada). The constructed Phylogenetic tree showing the clustering relationship among coliphages produced five major groups as shown in Figure 6. They were assigned their accession numbers; MK370060, MK370061 & MK370062, respectively.

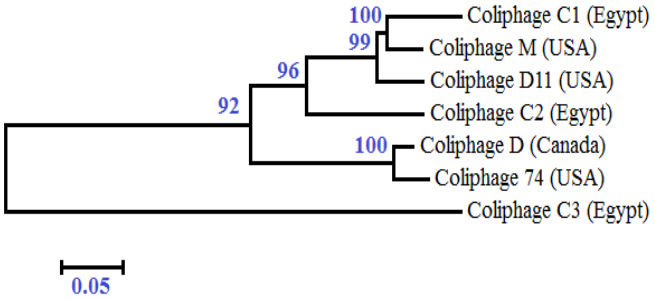

Fig. 6: Neighbor-Joining tree of newly Egyptian phages and other phages published in GenBank. Numbers represent bootstrap percentage values based on 1000.

On the other hand, all nucleotide of CP gene sequences were translated and coded protein were designed by 3D models structures for the three coliphages particles. Our results showed that different amino acids and three distinct protein types were constructed as illustrated in Figure 7.

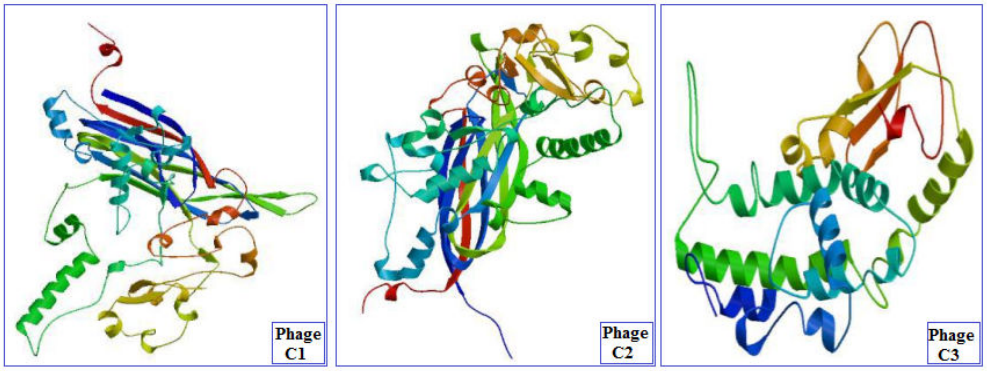

Fig. 7: Protein homology modeling designed using Swiss model of three Egyptian phage isolates (C1, C2 and C3), respectively.

Based on amino acid sequences of the major coat protein gene, a phylogenetic tree was constructed and produced three major groups as shown in Figure 8.

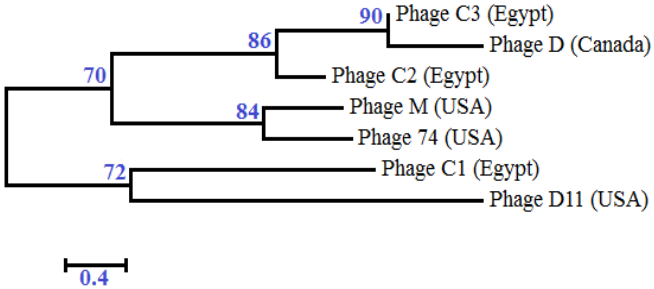

Fig. 8: Phylogenetic tree of coliphage isolates and related phages submitted in Genbank based on amino acid sequences.

As demonstrated previously, the genomic analysis provided a unique opportunity for comparative illustration of nucleotide diversity among novel coliphages isolated from Egyptian aquatic environment and other geographically distant coliphages submitted in GenBank, and comprehensively reflects the novelty of phage stock employed in the present study.

Molecular identification of bacterial host

Molecular identification techniques are becoming the method of choice in environmental microbiology, owing to circumvent the problem of phenotypes variability and overwhelm the obstacle of species misidentification (Ramirez-Castillo *et al.,* 2015). Rapid detection and identification of *E.coli* in water traced in our investigation included selection of three strains (A1, A2 & A3) for 16S-rDNA sequence analysis, based on their recognizable positive results in plaque assay with phage stock as well as purity and quantity of DNA yield.

Multiple sequence alignment (MSA) was displayed to compare the nucleotide sequences of the three Egyptian strains; A1 (252 bp), A2 (254 bp) and A3 (217 bp) with other international strains. The nucleotide sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database, USA. They were assigned the accession numbers MK359106.1, MK359107.1 and KY3591367.1, respectively.

Aquatic *E.coli* strains detected by proposed phage stock were 100% confirmed by16S rDNA based PCR assay. Our results agree with those reported by Bergeron *et al.,* (2011) who mentioned that, the potential for misidentification of *E.coli* in water using molecular methods were nearly negligible. Meanwhile, conventional cultural methods could hamper identification of contamination sources and implementation of effective control measures. Molecular methods mediated superior specificity and sensitivity than conventional phenotypic diagnostic tests with percentages reaching 90-100% accuracy in similar studies (Deshmukh *et al.,* 2016 and Olowe *et al.,* 2017).

On the other hand, the phylogenetic tree was constructed and showed the genetic relationship between Egyptian *E.coli* (A1, A2 & A3) strains and other submitted international strains from GenBank according to sequence similarity values. Five clusters are clearly demonstrated in **Figure 9** in which, strains A1, A2 and A3 showed 82% homology with each other and 99% homology with strains R1 and PSB7. According to given accession numbers, the three strains were found to be highly homologous (80-99%) with strains from Iran, India, Saudi Arabia and France.

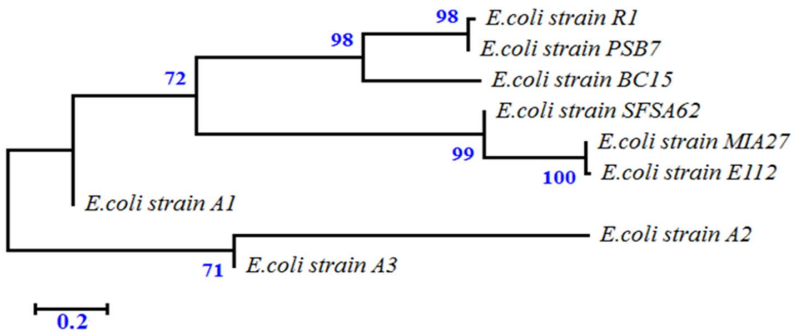

Fig. 9: Neighbour-joining phylogenetic tree of bacteria based on 16S rRNA gene sequence comparisons. Bootstrap values from 1000 replications are indicated at the branches.

Our results indicate observable genetic variability among *E.coli* strains detected by plaque assay. This definitely reflects the broad spectrum ability of coliphages employed in this study to target a wide array of environmental *E.coli* strains in water as much as possible, and supports its high specificity (95%) concluded from earlier statistical analysis. Thus, it is more advantageous to get benefit from the synergistic effect of more than one phage rather than using them individually (Al-Mola and Al-Yassari, 2010 and Dubey *et al.,* 2015).

Although the initial bacterial concentration is considered a major contributing factor governing the likelihood of a positive response, yet the number of input phages established in this study was

maximized to increase the probability of phage/bacterium hit. Accordingly, our future vision and efforts are depicted to maximize the number of newly isolated and characterized phages from aquatic environments. This approach could open new prospects for direct detection of pathogens from water specimens without the need to isolate pure bacterial cultures. Our vision is consistent with Askora *et al.,* (2015); Maal *et al.,* (2015) and Ribeiro *et al.,* (2018).

Conclusions

Our study confirms the importance of bacteriophage-based assays applied by plaque assay technique using a novel coliphages in aquatic environment. Although molecular methods provide rapid alternatives to culture, their wide-spread use is limited by cost, infrastructure requirements and the need for skilled operators. Various bacteriophage-based assays have been investigated for various and specific bacterial host detection. These assays offer the advantages of rapid, sensitive and specific host detection, cheap production costs as well as detecting only viable cells. Future research on bacteriophage diagnostics could yield assays capable of detecting and discerning between multiple bacterial pathogens.

References

Al-Mola, G.A. and M.A. Al-Yassari, 2010. Characterization of *E.coli* phage Isolated from Sewage. Al-Qadisiya J. of Vet. Med. Sci., 9 (2), 12-23.

Amarillas, L., L. Rubí-Range, C. Chaidez, A. González-Robles, L. Lightbourn-Rojas and J. LeónFélix1, 2017. Isolation and characterization of phills, a novel phage with potential biocontrol agent against multidrug-resistant Escherichia coli. Front Microbiol., 8,1355-1377.

American Public Health Association (APHA) and American Water Works Association and Water Environment Federation, 2012. Standard Methods for the Examination of Water and Wastewater, 22nd edition. American Public Health Association, Washington, DC.

Askora, A., A. Merwad, R.M. Gharibe, and A.I. Maysa, 2015. A lytic bacteriophage as a biocontrol for some enter pathogenic and enter hemorrhagic Escherichia coli strains of zoonotic risk in Egypt. Revue Med. Vet., 166(34):76-83.

Azzam, M.I., S.M. Ezzat, B.A. Othman and K.A. El-Dougdoug, 2017. Antibiotics resistance phenomenon and virulence ability in bacteria from water environment. Water Science, 31(2):109-121.

Balière, C., A. Rincé, J. Blanco, G. Dahbi, J. Harel, P. Vogeleer, J.C. Giard and P. Mariani-Kurkdjian, 2015. Prevalence and characterization of Shiga toxin-producing and enteropathogenic Escherichia coli in shellfish-harvesting areas and their watersheds. Front Microbiol., 6, 1356.

Bergeron, P., H. Oujati, V. Cuenca, J.Huguet Mestre and S. Courtois, 2011. Rapid monitoring of Escherichia coli and Enterococcus spp. in bathing water using reverse transcription-quantitative PCR. A key priority in prevention of nosocomial infection. Inter. J. of Hygiene and Environ. Health, 6,478-484.

Brenner, D.J., N.R. Krieg and J.T. Staley, 2005. Bergy's Manual of Systematic Bacteriology, 2nd edition. V. (2), the Proteobacteria. Springer, New York.

Chandran, A. and A. Mazumder, (2015). Pathogenic potential, genetic diversity and population structure of Escherichia coli strains isolated from a forest-dominated watershed (Comox Lake) in British Columbia, Canada. Appl. Environ. Microbiol., 81,1788-1798.

Chen, Z., D. Yu, S. He, H. Ye, L. Zhang, Y. Wen, W. Zhang, L. Shu and S. Chen, 2017. Prevalence of antibiotic-resistant Escherichia coli in drinking water sources in Hangzhou City. Front Microbiol., 1(8), 1133-1144.

Clokie, M.R. and A.M. Kropinski, 2009. Bacteriophages Methods and Protocols: V. (1): Isolation, Characterization and Interactions. Humana Press.

Dale, A.P. and N. Woodford, 2015. Extra-intestinal pathogenic *Escherichia coli* (ExPEC): disease, carriage and clones. J. Infect., 71, 615-626.

Dalmasso, M., R. Strain and H. Neve, 2016. Three new *Escherichia coli* phages from the human gut show promising potential for phage therapy. PLoS One, 11:e0156773.

Dubey, K., S. Chandraker, S. Sao and A. Gupta, 2015. Isolation and characterization of virulent coliphages from sewage sample. Int. J. Curr. Microbiol. App. Sci., 4(12), 699-704.

Dallal1, M.S., S.M Imeni, F. Nikkhahi, Z. Rajabi and S.P. Salas, 2016. Isolation of *E.coli* bacteriophage from raw sewage and comparing its antibacterial effect with ceftriaxone antibiotic. International Journal of Advanced Biotechnology and Research, 7(3), 385-391.

Deshmukh, R.A., K. Joshi, S. Bhand and U. Roy, 2016. Recent developments in detection and enumeration of water born bacteria: a retrospective minireview. Microbiology Open 5 (6), 901-922.

Donia, N., 2005. Rosetta branch waste load allocation model.19th International Water Technology Conference, Sharm El-Sheikh, Egypt. PP: 277-288.

El Gohary, R., 2015. Agriculture, industry and wastewater in the Nile Delta. Int. J. Sci. Res. Agric. Sci., 22,159-172.

Edgar, R., M. Mckinstry, J. Hwang, A.B. Oppenheim and R.A. Fekete, 2006. High-sensitivity bacterial detection using biotin-tagged phage and quantum-dot nanocomplexes. PANS, 103(13), 4841-4845.

Ettenauer, J., K. Zuser, K. Kellner, T. Posnicek and M. Brand, 2015. Development of an automated biosensor for rapid detection and quantification of *E.coli* in water. Procedia Engineering, 120, 376-379.

Ezzat, S.M., H.M. Mahdy, M.A. Abo-State, E.H. Abd El Stakour and M.A. El-Bahnasawy, 2012. Water quality assessment of River Nile at Rosetta branch: Impact of drains discharge. Middle East Journal of Scientific Research, 12 (4), 413-423.

Farrokh, C., K. Jordan, F. Auvray, K. Glass, H. Oppegaard, S. Raynaud, D. Thevenot, R. Condron, 2013. Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their significance in dairy production. Int. J Food Microbiol., 162,190-212.

Funatsu, T., T. Taniyama, T. Tajima, H. Tadakuma and H. Namiki, 2002. Rapid and sensitive detection method of a bacterium by using a GFP reporter phage. Microbiol. Immunol., 46, 365-369.

George, J., L. Divya and S. Suriyanarayanan, 2013. Quantitative microbial risk assessment in the management of *Escherichia coli* strains via drinking water. Journal of Environmental Research and Development, 8(1), 60-66.

Gould, L.H., 2012. Update recommendations for diagnosis of shiga toxin-producing *Escherichia coli* infections by clinical laboratories. Clin. Microbiol. Newslett., 34(10), 75-83.

Griffiths, M.W., 2010. Phage-based methods for the detection of bacterial pathogens. In: Sabour, P.M., Griffiths, M.W., eds. Bacteriophages in the control of food and waterborne pathogens. Washington, D.C: ASM press.

Health Canada, 2010. Guide lines for Canadian Recreational water quality 3rd ed, Health Canada, Ottawa, Ontario.

Jamalludeen, N., A.M. Kropinski, R.P. Johnson, E. Lingohr, J. Harel and C.L. Gyles, 2008. Complete genomic sequence of bacteriophage ϕEcoM-GJ1, a novel phage that has *Myovirus* morphology and a *Podovirus*-like RNA polymerase. Appl. Environ. Microbiol., 74, 516-525.

Jamal, M., T. Hussain, C. Rajanna Das and S. Andleeb, 2015. Isolation and characterization of a *Myoviridae* MJ1 bacteriophage against multi-drug resistant *Escherichia coli* 3. Jundishapur J Microbiol., 8(11), 1-8.

Jang, J., G. Hur, J. Sadowsky, M. Byappanahalli, T. Yan and S. Ishii, 2017. Environmental *Escherichia coli*: Ecology and public health implications. Journal of Applied Microbiology, 123, 570-581.

Joyanes, P., M. Conejo, L. Martinez and E.J. Perea, 2001. Evaluation of VITEK2 system for theidentification and susceptibility testing of three species of non-fermenting Gram-negative rods frequently isolated from clinical samples. J. Clin. Microbiol., 39, 3247-3253.

Jukes, T.H. and C.R. Cantor, 1969. Evaluation of Protein Molecules. In: Mammalian Protein Metabolism, Munro, H.N. (Ed.) Academic Press, New York, pp. 21-132.

Juang, D.F. and J.M. Morgan, 2001. The applicability of the API 20E and API rapid net systems for the identification of bacteria from activated sludge. Electron. Biotechol., 4(1), 1-7.

Kohn, T., M. Mattle, M. Minella and D. Vione, 2016. Modeling approach to estimate the solardisinfection of viral indicator organisms in waste stabilization ponds and surface waters. Water Research, 88, 912-922.

Lavigne, R., P. Darius, E.J. Summer, D. Seto, P. Mahadevan, A.S. Nilsson, H.W. Ackermann and A.M. Kropinski, 2009. Classification of *Myoviridae* bacteriophages using protein sequence similarity. BMC Microbiol., 9,224.

Li, N., G. Sheng, Y. Lu, R. Zeng and H. Yu, 2017. Removal of antibiotic resistance genes from wastewater treatment plant effluent by coagulation. Water Research, 111, 204-212.

Litt, P.K., J. Saha and D. Jaroni, 2018. Characterization of bacteriophages targeting Non-O157 Shiga toxigenic *Escherichia coli*. J. Food Prot., 81, 785-794.

Liu, H., H. Geagea, G.M. Rousseau, S.J. Labrie, D.M. Tremblay, X. Liu and S. Sylvain Moineau, 2018. Characterization of the *Escherichia coli* virulent Myophage ST32. Viruses, 10(11), 616.

Luhtanen, A.M., E. Eronen-Rasimus, H. Kaartokallio, J.M. Rintala, R. Autio and E. Roine, 2014. Isolation and characterization of phage host systems from the Baltic Sea ice. Extremophiles, 18(1), 121-130.

Maal, K.B., A.S. Delfan and S. Salmanizadeh, 2015. Isolation and Identification of two novel *Escherichia coli* bacteriophages and their application in wastewater treatment and coliforms phage therapy. Jundishapur J. Microbiol., 8(3), 1-7.

Maheux, A.F., L. Bissonnette, M. Boissinot, J.T. Bernier, V. Huppé, F.J. Picard, E. Bérubé, M.G. Bergeron, 2011. Rapid concentration and molecular enrichment approach for sensitive detection of *Escherichia coli* and *shigella* species in potable water samples. Appl. Environ. Microbiol.,77(17), 6199-6207.

Mohan Raj, J.R., R. Vittal, P. Huilgol, U. Bhat and I. Karunasagar, 2018. T4-like *Escherichia coli* phages from the environment carry blaCTX-M. Lett. Appl. Microbiol., 67, 9-14.

Millar, M., J. Seale and J. Turton, 2016. Prevalence of *Escherichia coli* virulence genes in patients with diarrhea and a subpopulation of healthy volunteers in Madrid, Spain. Front Microbiol., 7,11747.

Miyazaki, K., M. Sato and M. Tsukuda, 2017. PCR primer design for 16S rRNAs for experimental horizontal gene transfer test in *Escherichia coli*. Front Bioeng Biotechnol., 5,14-21.

Nasr, M. and H. Zahran, 2015. Assessment of agricultural drainage water quality for safe reuse in irrigation applications a case study in Borg El-Arab, Alexandria. J. Coastal Life Med., 3(3), 241- 244.

Olowe, B.M., J.O. Oluyege, O. Famurewa, A.O. Ogunniran and O. Adelegan, 2017. Molecular identification of *Escherichia coli* and new emerging enteropathogen, *Escherichia fergusonii*, from drinking water sources in Ado-Ekiti, Ekiti State, Nigeria. Journal of Microbiology Research, 7(3), 45-54.

Periasamy, D. and A. Sundaram, 2013. A novel approach for pathogen reduction in wastewater treatment. J. Environ. Health Sci. Eng., 11(1), 12.

Ramirez-Castillo, F.Y., A. Loera-Muro, M. Jacques, P. Garneau and F.J. Avelar-Gonzalez, 2015. Waterborne pathogens: Detection methods and challenges. Pathogens, 4, 307-334.

Ribeiro, K.V., C. Ribeiro, R.S. Dias, S.A. Cardoso, S.O. de Paula, J.C. Zanuncio and L. de Oliveira, 2018. Bacteriophage isolated from sewage eliminates and prevents the establishment of *Escherichia coli* biofilm. Adv. Pharm. Bull., 8(1), 85-95.

Sanger, F., S. Nicklen and A. Coulson, 1977. Biochemistry DNA sequencing with chain terminating inhibitors (DNA polymerase/ nucleotide sequences/ bacteriophage 4x147). Proc. Nati. Acad. Sci. USA., 74(12), 5463-5467.

Schofield, D.A., N.J. Sharp and C. Westwater, 2012. Phage-based platforms for the clinical detection of human bacterial pathogens. Bacteriophage, 2(2), 105-117.

Smart, A.E. and S. Ripp, 2011. Bacteriophage reporter technology for sensing and detecting microbial targets. Anal Bioanal Chem., 400, 991-1007.

Song, B., H. Xiong and Y. Xu, 2007. Isolation of a novel polyvalent virulent bacteriophage of *E.coli*. J Med Coll PLA., 22, 2617.

Stephenson, F.H., 2010. Calculations for molecular biology and biotechnology, 2nd ed., California, pp. 83-98.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar, 2013. MEGA 7: molecular evolutionary genetic analysis (MEGA) software version 7.0 Mol. Biol. Evol., 30(12), 2725-2729.

Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. Clustalw. Improving the sensitivity of progressive multiple sequences alignment through sequencing weighting, positions specific gap penalties and weight matrix choice. Nucleic Acids Res., 22, 4673-4680.

Um, M., O. Barraud, M. Kérourédan, M. Gaschet, T. Stalder and E. Oswald, 2016. Comparison of the incidence of pathogenic and antibiotic-resistant *Escherichia coli* strains in adult cattle and veal calf slaughterhouse effluents highlighted different risks for public health. Water Res., 88, 30-38.

Valério, N., C. Oliveira, V. Jesus, T. Branco, C. Pereira, C. Moreirinha and A. Almeida, 2017. Effects of single and combined use of bacteriophages and antibiotics to inactivate *Escherichia coli*. Virus Res., 15(240), 8-17.

Williams, I.G., A.R. Kubelik, K.J. Livak and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18, 6231-6235.

Yahya, M., F. Hamaied, S. Jebri, J. Jofre and M. Hamdi, 2015. Bacteriophages as indicators of human and animal fecal contamination in raw and treated wastewaters from Tunisia. J. Appl. Microbiol. 118, 1217-1225.

Yousry, M., A. El-Sherbini, M. Heikal and T. Salem, 2009. Suitability of water quality status of Rosetta branch for West Delta water conservation and irrigation rehabilitation project. Water Sci., 46, 47-60.

Wu, L., T. Huarg, L. Yang, J. Pan, S. Zhu and X. Yan, 2011. Sensitive and selective bacterial detection using tetracysteine-tagged phages in conjunction with biarsenical dye. Angew. Chem., 123, 5995-5999.

Wu, B., R. Wang and A. Fane, 2017. The roles of bacteriophages in membrane-based water and wastewater treatment processes: A review. Water Research, 110, 120-132.