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An approach using a novel phage mix for detecting *Pseudomonas aeruginosa* in water

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Abstract

The present study aims to integrate the benefits of plaque assay using a novel phage mix with phylogenetic and molecular analysis for detecting *Pseudomonas aeruginosa* in water. Three phages were isolated and the transmission electron microscope related their morphological resemblance to those of *Siphoviridae* and *Podoviridae* families, while molecular analysis showed different cp-gene sizes. The Phage mix was highly specific (86.0%), and data misleading didn't exceed 14.0% compared to membrane filter assay (39.2%). Time elapsed for test completion required 24 h. Identified *P. aeruginosa* were verified using 16S-rDNA. Nucleotide sequence data for both phages and bacteria were submitted to the NCBI GenBank database, USA and gained their accession numbers. Concluding remarks highlight the potential of plaque assay as specific, simple and rapid method. The study recommended future efforts to isolate and characterize new phages for detecting other bacterial pathogens of public health concern to control water pollution and maintain adequate hygiene.

Introduction

Microbial pollution is considered one of the most important challenges facing water quality status, not only in Egypt but also worldwide. Diseases experienced through contaminated water resources kill about five million children annually and contribute to one-sixth of the world population sickness (WHO/UNICEF, 2004). Unfortunately, approximately 663 million people worldwide live without access to safe water. Furthermore, in developing countries, 17% of all deaths of children below 5 years of age are usually following ingestion of poor water quality (WHO/UNICEF, 2015).

Pseudomonas aeruginosa is a Gram-negative bacterium that is highly ubiquitous in diverse ecological niches, such as soil and water ecosystems (Streeter and Katouli, 2016). It has been detected and isolated from different water resources, including rivers and lakes (Mena and Gerba, 2009), wastewater and sewage treatment plants (Ezzat *et al*., 2014), and public recreational swimming pools (Lutz and Lee, 2011) as well as tap water and bottled water (Zhang *et al*., 2013).

This organism enjoys notable virulence traits including minimal nutritional requirements, which contribute to its broad adaptability and environmental diversity (Ullah *et al*., 2012). Indeed, such profile reflects a genome of large size and complexity compared with other bacterial colleagues.

The most impressing feature of public health concern is related to its categorization as a significant human opportunistic pathogen (Driscoll *et al*., 2007; Streeter and Katouli, 2016); *P. aeruginosa* is responsible for about 10% of total hospitals acquired infections (Morales *et al*., 2012). It is responsible for 8% of surgical wound infections, 10% of blood infections, 12% of urinary tract infections and 16% of pneumonia cases. The problem becomes challenging due to its high resistance to a wide range of antibiotics (Adeola and Babatunde, 2016). Recent studies have demonstrated that effluents from wastewater significantly harbor antibiotic resistance genes that have become a threat to human health (Li *et al*., 2017). In this respect, significant average mortality rates of up to 1400 deaths per year have been reported. The common route of acquiring such infections was contaminated water (Asghari *et al*., 2013).

In response to the aforementioned hazards, minimizing exposure of high-risk patients by eliminating contamination sources underscores the importance of fast and reliable detection methods of this bacterium in water systems. Conventional methods encountered as tools for detecting water safety regarding microbial pollution have been applied worldwide; however, limitations concerning

data accuracy and reproducibility are questioned nowadays. These methods, including membrane filter assay, culture techniques and biochemical testing are time consuming and labor intensive, both for setup and ongoing evaluation, generally requiring not less than 3–4 days for completion. Furthermore, they may fail to detect bacteria that become non-culturable in response to environmental stress (Al-Qadiri *et al*., 2006; Khattab *et al*., 2015).

Within this context, phage-based diagnostics could have the potential to fulfill these gaps and overwhelm the problem through rapid, sensitive, and cost effective detection of its specific host in a variety of environmental matrices. Extensive evidences support this notion, because bacteriophages (virus infecting bacteria) possess a number of desirable attributes which make them suited as unique bacterial detectors. Bacteriophages are characterized by their abundance in water and wastewater, their inherent natural specificity, ease of use and straightforward production (Wu *et al*., 2017). Typically, a single phage particle can attack only specific strain of targeted bacterial species, and host specificity is usually determined by outer proteins (receptors) of the bacterium to which a phage attaches during the initial infection process. In nature, once the phage finds its specific receptors, it binds to the bacterial cell and injects its nucleic acid. The rapid phage replication finally mediates the way for bacterial cell wall lysis (Wu *et al*., 2011).

Despite these attributes, there are surprisingly very few phage-based diagnostics that have been transitioned from research lab to current use. The most important of which are for detecting *E. coli* O157:H7, *Mycobacterium tuberculosis*, *Yersinia pestis*, *Bacillus anthracis* and *Staphylococcus aureus* (Schofield *et al*., 2012). They include: plaque assay, phage amplification, reporter phage, phage-labelling and phage capture elements (Funatsu *et al*., 2002; Griffiths, 2010; Smart and Ripp, 2011; Nouraldin *et al*., 2016).

In 1988, the National Center for Biotechnology Information (NCBI) was established by US Government to host all composite data base and information related to molecular biology. It provides interconnections between data of genetics and proteins sequences, phylogenetic tree based data, genomes and literature references (Benson *et al*., 2012). It also includes nucleotides sequences database called GenBank which covers genome sequences of more than 340 000 formally described species that can be retrieved by the NCBI's integrated retrieval system (Clark *et al*., 2016). Based on the rapid development in the field of bioinformatics, the upcoming years could help us to open new computational areas in the field of water quality assessment to save our time, energy and costs and guarantee more accurate and reliable data.

In response to previous challenges, the present study was conducted to evaluate the efficacy of a newly isolated phage mix for detecting *Pseudomonas aeruginosa* in water, being a model index for water born opportunistic pathogen of public health concern. The potentials of Phylogenetic and molecular analysis were invested to verify the detected bacteria and their phages. Superiority and reliability of these techniques compared to traditional methods were also investigated.

Materials and methods

Study area and sampling procedure

The area covered 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 Fig. 1. Totally 15 sites were chosen, 3 from each reach: 5 at drains outfalls (El-Rahway, Sabal, El-Tahreer, Zawiet El-Bahr and Tala) and 10 sites in Rosetta branch (5 upstream and 5 downstream those drains outfalls). These are mixed drains from sewage, agricultural and industrial wastes.

The study was started with samples collection in which 90 water samples were collected in duplicates, 60 from Rosetta branch and 30 from drains outfalls as described above. Samples collection was processed in two different seasons (Summer & Winter, 2015–2016) 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 they have been analysed.

Detection of P. aeruginosa in water samples

Membrane filter assay, phage-based diagnostic, phylogenetic and molecular analysis were applied simultaneously as follows:

Membrane filter assay

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 0.45 µm which retained bacteria. After filtration, the membrane was plated on BBL™ *M*-PA-C Agar and incubated at 41.5°C/72 h. Results were recorded as colony forming unit (CFU/100 ml) using the following equation:

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

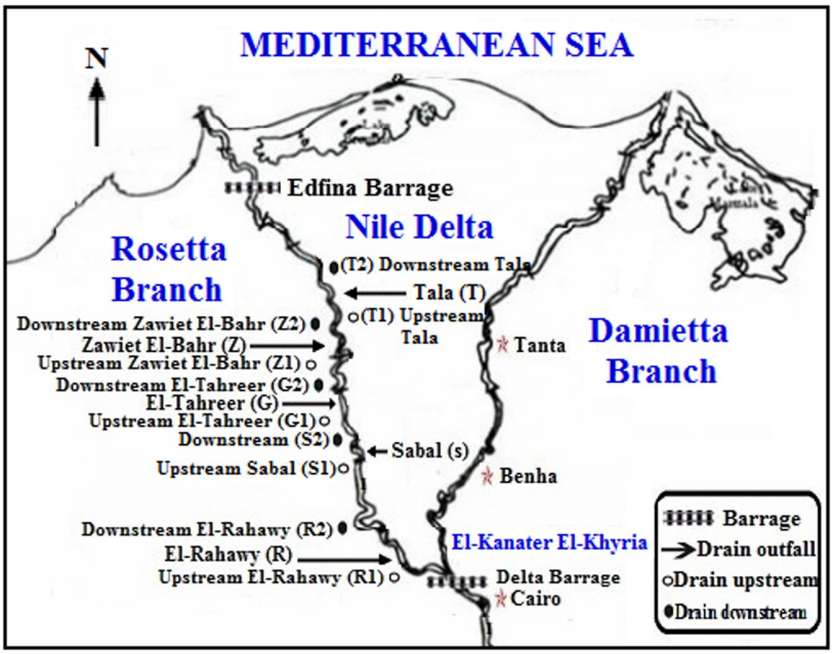

Fig. 1. Map of sampling locations (Drains and Rosetta branch). [Colour figure can be viewed at wileyonlinelibrary.com]

These colonies were confirmed by streaking on Difco™ Cetrimide Agar Base plates, a selective medium which inhibits bacterial growth except *P. aeruginosa* and enhances fluorescein and pyocyanin 'blue green' pigment production. 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.

Phage isolation

Bacteriophages specific to *P. aeruginosa* were isolated from all water samples (Drains & Rosetta branch) according to method described by Cerveny *et al*. (2002) and Kumari *et al*. (2009). In this method, water samples were centrifuged at 10 000 rpm for 10 min at 4°C. The supernatants were filtered through sterile 0.45 µm pore size sartorious membrane. A mixture of 50 ml filtered water sample, 50 ml sterile nutrient broth and 5.0 ml overnight American Type Culture Collection *P. aeruginosa* (ATCC® 27853) was incubated at 37°C for 24 h. The mixture was centrifuged to remove bacteria and the supernatant was filter sterilized and checked for the presence of lytic phages using spot test.

Spot test

In this test, 10 µl of obtained supernatant was spotted onto lawns of *P. aeruginosa* (ATCC® 27853) cultured on tryptone soya agar plates. Formation of clear zones after overnight incubation suggested the presence of lytic phages specific for host strain (Nouraldin *et al*., 2016).

Plaque assay

The phage titer was determined quantitatively using double agar overlay (plaque assay) as originally described by Adams (1959). Phage suspensions obtained from all water samples were serially diluted (tenfolds), and 100 µl from each dilution were added to 100 µl host bacterium, then mixed with 5.0 ml sterile molten soft (0.7% agar w/v). The mixture was poured quickly on top of solidified nutrient agar plates and left to harden for 30 min then incubated at 37°C. *P. aeruginosa* (ATCC® 27853) was used as reference host bacterium at concentration 10^8 CFU/ml. After only 24 h, plates were checked for plaques formation, and plaque forming units were calculated according to Stephenson (2010) using the following equation:

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

Phages resulting from plaque assay were further purified as described by Sambrook *et al*. (1989) and Clokie and Kropinski (2009). Separated single plaques were picked up based on their morphological variability and re-plated three times to ensure pure phage stocks. These phages were further inoculated into 5.0 ml nutrient broth and incubated at 37°C after mixing with 1% overnight culture of host strain. After complete lysis, the mixture was centrifuged, filter sterilized and treated with chloroform (1% w/v) to remove any bacterial cells. Purified phages were stored in 60% glycerol at –80°C for long term storage, while short term stocks were maintained at 4°C for further investigations.

Phage specification

The practicability of rapid detection of *P. aeruginosa* in water using plaque assay was evaluated experimentally. Typical *P. aeruginosa* (107 isolates) from various water sources (Drains and Rosetta branch) were employed to determine the specificity of isolated phage mix stock and ensure the ability of these phages to target as many *P. aeruginosa* strains as possible. Again, the double agar layer plaque was applied as described above and *P. aeruginosa* (ATCC® 27853) was used as positive control for the test, while *E. coli* (ATCC® 25922) was used as negative control.

Transmission electron microscopy (TEM)

The isolated stock phages were examined for morphological characterization using TEM model Beckman 1010 at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Molecular characterization of isolated phages

Based on their cultural and morphological variability, three purified phages isolated from plaque assay were chosen for extraction and purification of total DNA. The DNeasy plant mini kit (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 were designed based on the morphological characterization of the three isolated phages which are similar to *Psedomonas* phage H66 and D3 recorded in GenBank under accession numbers KC262634.1 for Phage H66 (29674..30966) and NC_002484 for phage D3 (4459..5646). The specific primers were selected from six primer sets based on % GC and temperature annealing (T_m) as shown in Table 1.

PCR reaction was performed in 30 µl volume tubes according to Williams *et al*. (1990) that contained the following: dNTP (2.5 mM), 3.00 µl; $MgCl_2$ (25 mM), 3.00 µl; PCR-buffer (10X), 3.00 µl; Primer (10 P mol), 2.00 µl; Taq DNA polymerase (5u/ml), 0.20 µl; Template DNA (25 ng), 2.00 µl; d.H20, 16.80 µl 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 10 min.

Table 1 Specific primer sets of *P. aeruginosa* phages

Primers	Sequence	T_m (°C)	% GC	Expected size (bp)
Forward	5′ ACGCCGAGAAGAAGACCAAG 3′	60.04	55.00	1261
Reverse	5′ ATCAGGGTGTTGCTCCACAG 3′	60.02	60.00	
Forward	5′ CCCTGCAGAGCTACATCGAC 3′	60.25	55.00	1141
Reverse	5′ GAACTCGTCCTGGGTGATGG 3′	60.11	60.00	
Forward	5′ AAGAACGGCGACGAGAACAT 3′	60.04	50.00	961
Reverse	5′ ACTTGTCCCAGTTGGTGCTG 3′	60.47	60.00	

Sequencing for phages was carried out in MC Lab., USA (www.mclab.com), using the dideoxyribonucleoside chain termination procedure originally developed by Sanger *et al*. (1977). Nucleotide sequences were determined automatically by the electrophoresis of the cycle sequencing reaction product on 310 Genetic Analyzer. Data were provided as fluorometric 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 6.0) (Tamura *et al*., 2013).

Phylogenetic relationships among identified phages were evaluated using unweighting pair Group Method with Arithmetic Mean (UPGMA) through MEGA 6.0 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 to the National Center for Biotechnology Information (NCBI) GenBank database, USA, and assigned their accession numbers.

Verification of detected P. aeruginosa by16S-rDNA

P. aeruginosa isolates showing recognizable susceptibility to phage lytic infection were selected for 16S-rDNA

sequence analysis. Selection was based on purity of the DNA as well as PCR amplified product. Genomic DNA 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 using a Thermal cycler (ABI 2720) in 100 µl reaction volume containing 2.5 mM of dNTP, 10x PCR buffer, 3U of Taq DNA polymerase, 10 ng template DNA, and 400 ng of primer (F) 5'-GGGGGATCTTCGGACCTCA-3', and primer (R) 5'-TCCTTAGAGTGCCCACCCG-3' which were designed for *P. aeruginosa* according to Tripathi *et al*. (2013). The amplification program was set as an initial denaturation at 94°C for 5 min., followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min and a final extension at 72°C for 5 min. The sequencing was performed according to manufacturer's protocol using Big Dye Terminator Cycle Sequencing Kit (V. 3.1, Applied Biosystem) and analysed in an Applied Bio-system analyzer.

The sequences of 16S-rDNA for these strains were finally submitted to the NCBI GenBank database, USA, and compared to other available sequences using an automated alignment tool blast program, and assigned their accession numbers.

Phylogenetic tree showing the genetic relationship between the Egyptian strains obtained in this study and other recorded strains was constructed using Clustalw with the help of MEGA software version 6.0 (Tamoura *et al*., 2013).

Statistical and bioinformatics analysis

SPSS statistical software was used to calculate Min, Max and Mean values of measured parameters. Percentages and log transformed data were calculated for better illustration. The Pearson's correlation coefficient (r) was used to correlate true and false positive results of detected bacteria. For bioinformatics and molecular analysis, the following programs were employed in the study:

- Basic local alignment search tool (BLASTN and BLASTP).
- Clustalw program (version 1.74).
- Molecular Evolutionary Genetic Analysis (MEGA) software (version 6.0).
- DNAMAN software (Madison, Wisconsin, USA, version 5.2.9).

Results and discussion

Levels of P. aeruginosa recovered from membrane filter assay

Presumptive results showed that *P. aeruginosa* was detected in all water samples. The mean counts (CFU/100 ml) were expressed as 10 log CFU/100 ml for better data illustration and demonstrated as given in Fig. 2.

Gradual increase was recorded from upstream to downstream in Rosetta branch, presumably due to direct discharge from drains. Not surprisingly, maximum values (104–142 CFU/100 ml) were found at drains outfalls in summer and winter seasons, respectively. Meanwhile, levels in Rosetta branch downstream the drains were superior to those at upstream locations, and fluctuated between 65–89 CFU/100 ml at downstream and 16–38 CFU/100 ml at upstream, respectively, in summer and winter.

Similarly, earlier investigators detected the highest pollution levels in Rosetta branch downstream drains outfalls,

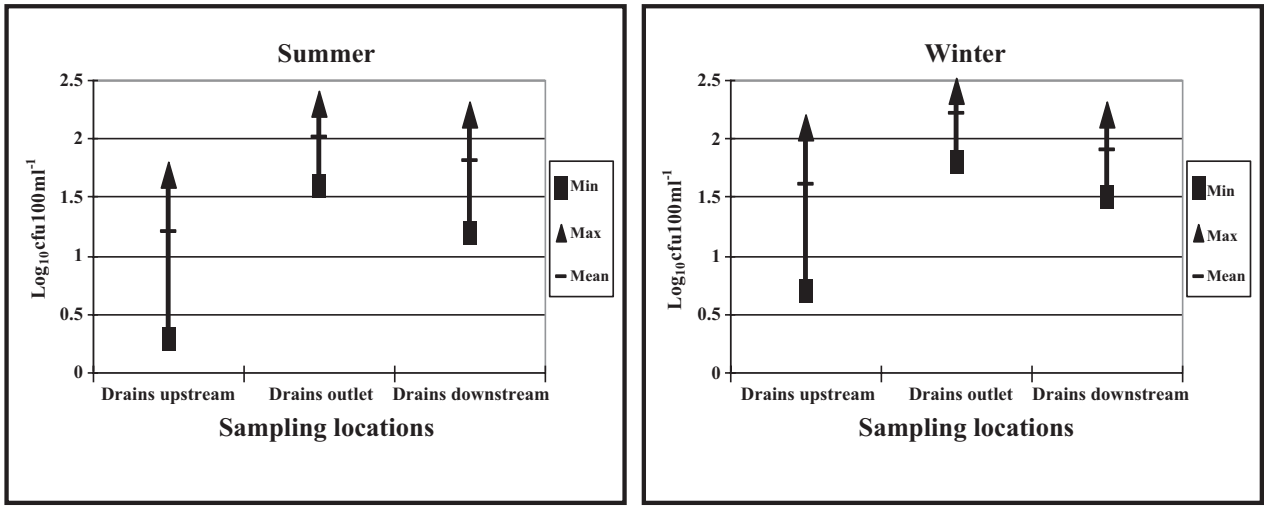

Fig. 2. Presumptive count of *P. aeruginosa* using membrane filter assays.

and addressed the problem to the combined influence of industrial, agricultural and domestic wastes discharge (Donia, 2005; Ezzat *et al*., 2012; Abo-State *et al*., 2012). In this respect, Health Canada has established guidelines for *P. aeruginosa* in water of ≤2 CFU/100 ml in case of primary human body contact, and ≤10 CFU/100 ml 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 in our study showed that, highest levels of *P. aeruginosa* 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).

The spatial distribution of *P. aeruginosa* 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. The same observations were also reported by Streeter and Katouli (2016).

Data confirmation and verification

In the present study, 90 water samples were processed in duplicates during two different seasons. Out of which, 176 presumptive *P. aeruginosa* colonies were isolated using membrane filter assay. These included 87 isolates (49.4%) from drains outlets, 56 isolates (31.8%) from drains downstream in Rosetta branch and 33 isolates (18.8%) from drains upstream in Rosetta branch.

These colonies were typically 0.8–2.2 mm in diameter and flat in appearance with light outer rims and brownish to greenish black centres. Streaked colonies on cetrimide agar showed enhanced fluorescein and pyocyanin 'blue green' pigment production, while microscopic examination revealed Gram-negative, non-spore forming bacilli. Phenotypic identification and verification were further processed using API 20NE assay.

As given in Table 2, only 107 isolates (60.8%) were confirmed as typical *P. aeruginosa*, meanwhile 69 (39.2%) were classified as being atypical colonies not belonging to *P. aeruginosa*. 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 0.99998. Data misidentification constituted about 39.2% and was considered to be non-ignorable bias, particularly when dealing with bacteria associated with human infections (Driscoll *et al*., 2007). Our results also matched those recorded by Altaai *et al*. (2014) who discovered 30% misidentification using classical techniques.

Table 2 Verified count of *P. aeruginosa* from membrane filter assays

	No. (%) of *P. aeruginosa* colonies	
Water samples	Typical	Atypical
Drains outlet	53 (60.9%)	34 (39.1%)
*RB before drains discharge	20 (60.6%)	13 (39.4%)
*RB after drains discharge	34 (60.7%)	22 (39.3%)
Total	107 (60.8%)	69 (39.2%)

*RB, Rosetta branch.

Based on principles learned from earlier investigations, the conventional culture methods and phenotypic identification systems could not solve the problems of marked phenotypic variability demonstrated by *P. aeruginosa* and other closely related species (Joyanes *et al*., 2001 and Qin *et al*., 2003). Furthermore, they usually fail to detect bacteria that become non-culturable due to environmental stress. Lag time needed (3–4 days) for test completion could certainly postpone identification of contamination source and implementation of effective control measures (Asghari *et al*., 2013; Deshmukh *et al*., 2016).

Detection of P. aeruginosa phages using plaque assay

Bacteriophages are the most abundant and diversified microorganisms on earth (Zhang *et al*., 2013; Zhan *et al*., 2015). Phages 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 (Chaturongakul and Ounjai, 2014; Li *et al*., 2016).

In the present investigation, the plaque assay was used as a phage-based diagnostic to detect the presence of *P. aeruginosa* phages in water samples. *P. aeruginosa* (ATCC® 27853) was employed as a reference host. Clearly, Fig. 3 demonstrates the incidence of *P. aeruginosa* phages in all tested water samples ($n = 90$), being maximum (10^5–10^6 pfu/ml) at drains outlets and minimum (10^2 pfu/ml) 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).

These findings coincide with our earlier results which recorded positive evidence for *P. aeruginosa* in all water samples with varying levels. Accordingly, the number and behaviour of phages were directly influenced by the densities of their receptive host. In this respect, phages have a high probability of encountering their host bacteria due to continual movement in water (Yahya *et al*., 2015). Supporting data from recent studies reported the

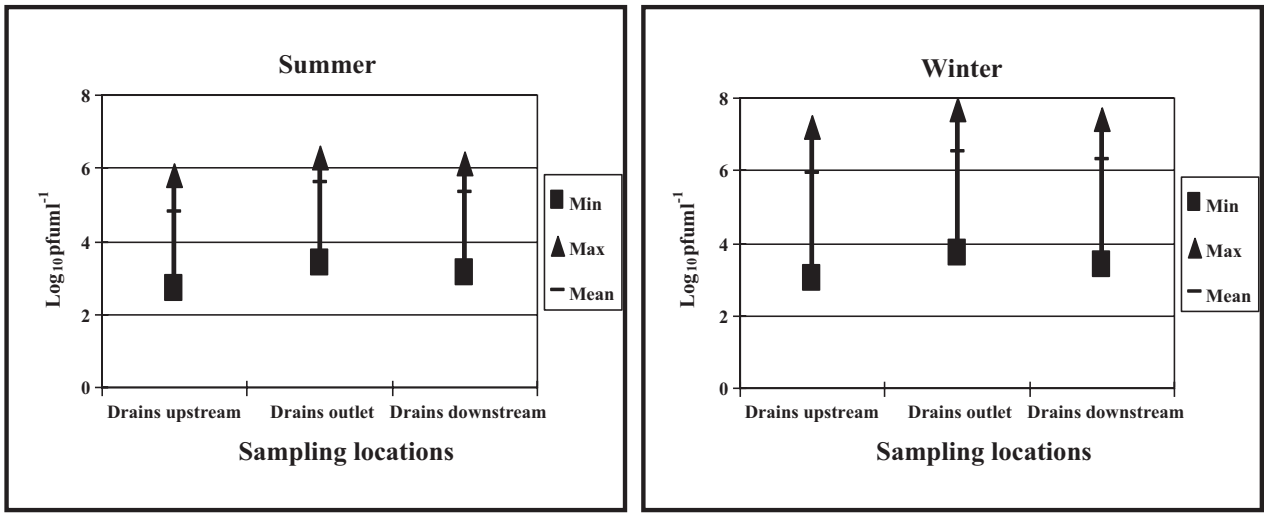

Fig. 3. Presumptive count of *P. aeruginosa* phages detected in water samples.

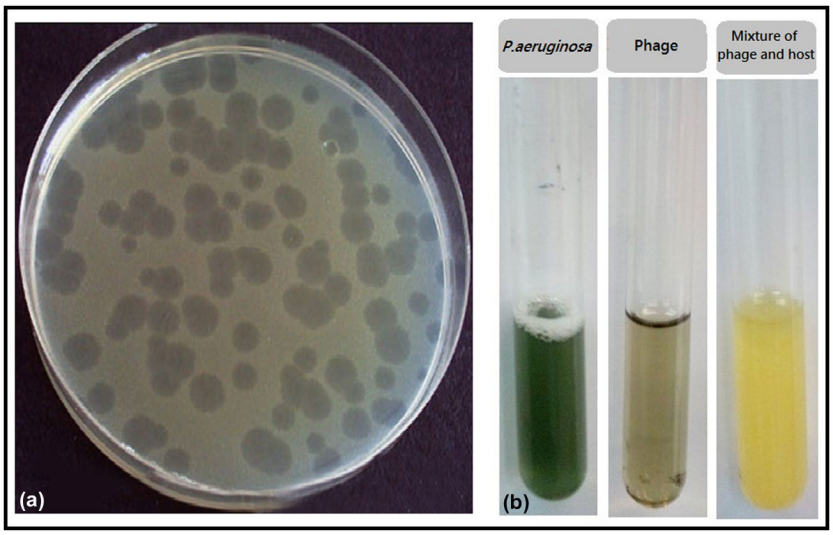

Fig. 4. Plaque assay after 24 h: (a) Plaques of *P. aeruginosa* phages; (b) Phage induced bacterial reduction. [Colour figure can be viewed at wileyonlinelibrary.com]

detection of *P. aeruginosa* phages, particularly from sewage water and aquatic ecosystems subjected to sources of microbial pollution (Seema *et al*., 2009; Omar *et al*., 2012; Zahra *et al*., 2014; Azizian *et al*., 2015; Nouraldin *et al*., 2016).

Figure 4(a) illustrates the diversity of plaques morphology developed by phages on *P. aeruginosa* lawns and expressed by clear hallow zones. The plaques size ranged between 2 and 5 mm in diameter, which were circular and clear either regular or irregular in morphology. Similarly, Han *et al*. (2014) and Sagar *et al*. (2015) detected and characterized *P. aeruginosa* phages of nearly comparable plaque sizes (2–4 mm diameter) from drainage water and Ganges river water, respectively. Consistent observation in our study reported dramatic decrease in *P. aeruginosa* cells on behalf of phage infection during reduction assay. The $O.D_{600}$ of bacterial titer (0.815 nm) dropped to 0.238 nm upon mixing with phage after 24 h of incubation (Fig. 4b).

Phage specification

The value of a phage diagnostic assay is mainly 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

Table 3 Susceptibility of *P. aeruginosa* strains to phage mix

Species	Source	No. of +ve results/No. of tested strains
P. aeruginosa	*ATCC 27853 (n = 1)	1/1 (100%)
	Drains outlet (n = 53)	49/53 (92.5%)
	**RB before drains discharge (n = 20)	15/20 (75.0%)
	**RB after drains discharge (n = 34)	28/34 (82.4%)
E. coli	*ATCC 25922 (n = 1)	0/1 (0%)

*American type culture collection (reference strains).
**RB, Rosetta branch.

host-susceptibility (Schofield *et al*., 2012). In this respect, phage cocktails may be used to overcome strains that are phage resistant and ensure a wider coverage within a given species. In the present study, an attempt has been made to evaluate the specificity and host range pattern of three isolated phage mix stock (PP01, PP02 and MS03) and ensure the ability of these wild-type phages to target as many *P. aeruginosa* strains as possible in water samples.

Table 4 Morphological features of phage isolates as determined by TEM

Phage isolates	Family	Head capsid (nm)		Tail (nm)	
		Length	Diameter	Length	Diameter
PP01	*Siphoviridae*	70 ± 4	70 ± 4	260 ± 7	20 ± 0
PP02	*Siphoviridae*	90 ± 3	90 ± 3	300 ± 10	20 ± 7
MS03	*Podoviridae*	60 ± 7	60 ± 7	35 ± 3	20 ± 0

As given in Table 3, among 107 typical *P. aeruginosa* isolates from various water sources (Drains and Rosetta branch) the phage mix was highly specific to 86.0% of tested strains compared to ATCC® 27853 as a positive control. Phage specificity reached about 92.5% in highly polluted samples (Drains outlets), followed by Rosetta branch after drains discharge (82.4%) and before discharge

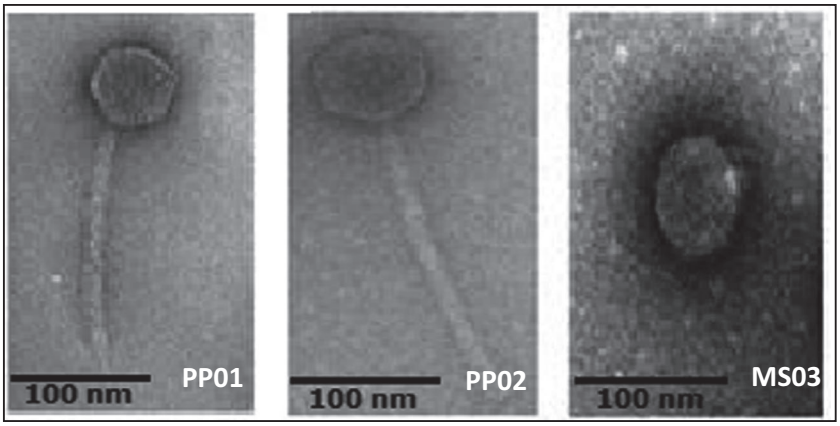

Fig. 5. Electron micrographs of negatively stained phage particles.

Table 5 Comparison between bases composition of complete cp-genes of isolated phages and five *Pseudomonas* phages published in GenBank

	Phage MS03	Phage PP02	Phage PP01	Phage LKA5	Phage F116	Phage KPP23	Phage PMG1	Phage Phi1
Bases composition	KY826473.1	KY797301.1	KY786116.1	KC900378.1	AY625898.1	AB910392.1	HQ711985.1	KT887557.1
Length (bp)	975	1185	1290	1290	477	978	1185	1185
Weight (kDa)	602.65	732.47	797.35	797.33	294.87	604.50	732.45	732.45
Adenine (A)	202	228	260	279	72	204	254	253
Cytosine (C)	345	434	465	447	185	337	400	402
Guanine (G)	312	395	415	413	157	320	404	402
Thymine (T)	116	128	150	151	63	117	127	128
C/G	657	829	880	860	342	657	804	804
A/T	318	356	410	430	135	321	381	381
Adenine (A)	20.7%	19.2%	20.2%	21.6%	15.1%	20.9%	21.4%	21.4%
Cytosine (C)	35.4%	36.6%	36%	34.7%	38.8%	34.5%	33.8%	33.9%
Guanine (G)	32%	33.3%	32.2%	32%	32.9%	32.7%	34.1%	33.9%
Thymine (T)	11.9%	10.8%	11.6%	11.7%	13.2%	12%	10.7%	10.8%
C/G	67.4%	70%	68.2%	66.7%	71.7%	67.2%	67.8%	67.8%
A/T	32.6%	30%	31.8%	33.3%	28.3%	32.8%	32.2%	32.2%

(75.0%). 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). It is worth mentioning that, 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 24 h 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); Zhang *et al*. (2013); Khawaja *et al*. (2016).

Transmission electron microscopy

Electron microscopy imaging of purified phage particles revealed different structural features and dimensions as demonstrated in Table 4 and Fig. 5.

The three phages are non-enveloped and have icosahedral capsid. PP01 and PP02 have long non-contractile tail, while MS03 has short non-contractile tail. According to International Committee on Taxonomy of Viruses (ICTV), the isolated phages belonged to the *Siphoviridae* and *Podoviridae* families. These two families have been documented to include many phages which have the ability

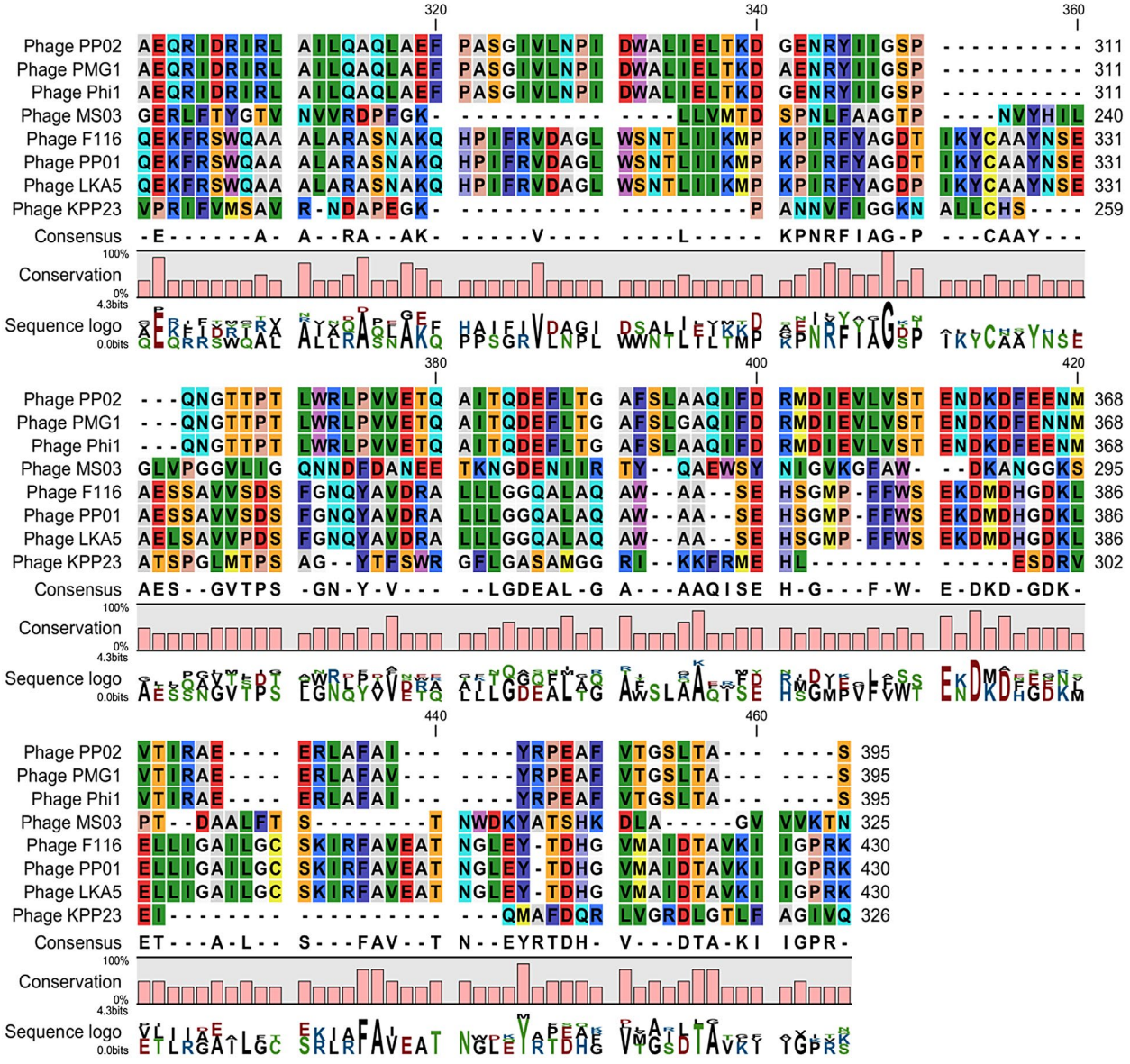

Fig. 6. Multiple amino acid sequences alignment of the cp-gene of three Egyptian phage isolates with the corresponding amino acid sequences of five *P. aeruginosa* phages available in GenBank. [Colour figure can be viewed at wileyonlinelibrary.com]

to infect members of Pseudomonadaceae (Luhtanen *et al*., 2014).

Genotypic identification of isolated phages

Results of DNA sequencing for the three virulent *P. aeruginosa* phages (PP01, PP02 and MS03) forming the phage mix showed different cp-gene sizes (1290, 1185p, and 975 bp), respectively. The cp-gene was chosen for genotypic identification because it is a principle component in phages and includes large number of nucleotides which are considered as conservative regions (Thomas *et al*., 2012).

The nucleotide sequences for the three phages were aligned with other coat protein sequences of *pseudomonas* phages published in the NCBI. They were assigned their accession numbers; KY786116.1, KY797301.1 and KY826473.1, respectively. The Multiple sequence alignment (MSA) revealed their genetic diversity compared with five geographically distant phages recorded in GenBank; Phage PMG1 (Accession No. HQ711985.1), Phage Phi1 (Accession No. KT887557.1), Phage F116 (Accession No. AY625898.1), Phage LKA5 (Accession No. KC900378.1) and Phage KPP23 (Accession No. AB910392.1). Comparisons between different bases composition of cp-genes were given in Table 5.

Furthermore, all nucleotide sequences for cp-genes were translated to amino acids and aligned as shown in Fig. 6. Phylogenetic tree was constructed and produced five clusters belonging to three major groups according to differences in genetic distances (Fig. 7). Similarities were recorded as follows; Phages PP02, Phi1 and PMG1 (93%), phages PP01 and LKA5 (85%), while phages MS03 and KPP23 (71%). The above bioinformatics analysis provides a unique opportunity for comparative illustration of nucleotide sequences diversity among phages isolated in this study and comprehensively reflects the novelty of employed phage mix.

Genotypic identification of detected P. aeruginosa

Genotype-based identification systems are becoming the method of choice in environmental microbiology, owing to circumvent the problems of phenotypes variability and species misidentification (Ramirez-Castillo *et al*., 2015).

Verification of detection and identification protocol of *P. aeruginosa* in water traced through this study involved selection of three strains (M1, M2 and M3) for 16S-rDNA sequence analysis, based on their recognizable positive results in plaque assay with phage mix as well as purity of DNA and PCR amplified products. Concentrations of extracted DNA were checked using spectrophotometer at wave length A_{260}/A_{280} giving values 1.6, 1.8 and 1.82

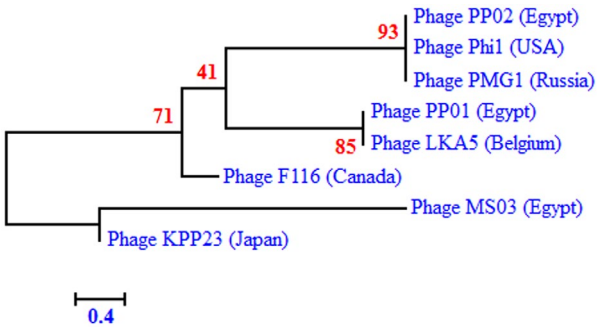

Fig. 7. Phylogenetic tree of phage isolates and related phages published in GenBank based on amino acid sequences of the cp-genes. [Colour figure can be viewed at wileyonlinelibrary.com]

for M1, M2 and M3, respectively. The PCR amplified products were run on 2% agarose gel electrophoresis to ensure purity giving three distinct fragments with different molecular weights; M1 (1246 bp), M2 (1253 bp) and M3 (1286 bp).

Multiple sequence alignment (MSA) was displayed to compare the nucleotide sequences of the three Egyptian strains with other strains from different localities. Nucleotide sequences were submitted to the NCBI GenBank database, USA, and were assigned the accession numbers LC094440.1, LC096954.1 and LC096955.1, respectively. Interestingly, these strains were 100% confirmed by16S-rDNA-based PCR assay.

Our results agree with those reported by Asghari *et al*. (2013) who mentioned that, the potential for misidentification of *P. aeruginosa* in water using molecular techniques 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 phenotypic diagnostic tests with percentages reaching 90–100% accuracy in similar studies (Altaai *et al*., 2014; Khattab *et al*., 2015; Deshmukh *et al*., 2016).

Based on MSA analysis, the phylogenetic tree was constructed to show the genetic relationship between *P. aeruginosa* (M1, M2 and M3) strains and other recorded strains from GenBank according to sequence similarity values. Twelve clusters are clearly demonstrated in Fig. 8 in which, strains M2 and M3 showed 79% homology with each other and 78% homology with M1. The three strains were found to be highly homologous (84%) with other geographically distant strains recorded in GenBank having the following accession numbers: NR026078.1, JF423918.1, XO6684.1, GU384267.1, HM439966.1 and LK391633.1.

The above results indicate observable genetic variability among *P. aeruginosa* strains detected by plaque assay.

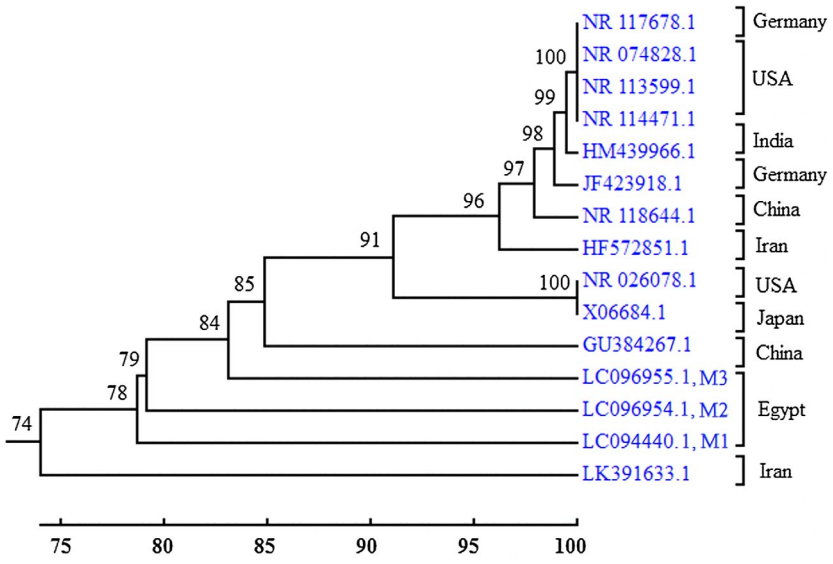

Fig. 8. Phylogenetic tree of *P. aeruginosa* strains based on 16S-rDNA gene sequences. [Colour figure can be viewed at wileyonlinelibrary.com]

This definitely reflects the broad spectrum ability of phage mix employed in this study to target a wide array of *P. aeruginosa* strains in water as much as possible, and supports its high specificity (86.0%) 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 (Cui *et al*., 2016).

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. 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 Danis-Wlodarczyk *et al*. (2015); Alsaffar and Jarallah (2016).

Conclusions

(1) The present investigation sums up the importance of phage-based diagnostics represented in our study by plaque assay using a novel phage mix. The method was proved to be specific, simple, and rapid for the detection of *P. aeruginosa* strains, being important water born opportunistic pathogen of public health concern.

(2) The inexpensive running costs and short term completion of this method provide a realistic tool in situations when funding is restricted and/or time is critically matter of concern. We believe that, one could apply this method to any given bacterial genus, as long as there is access to the appropriate natural phage.

(3) Feasibility of this approach was confirmed by molecular analysis, through which three *P. aeruginosa* strains and their specific phages were accessed in the NCBI. Future testing with larger number of phages is recommended, coupled with inter-laboratory validations.

(4) It could be possible to commercialize phage-based products as bacterial detectors for different water resources. Indeed, this could mitigate implementation of successful and rapid control measures of infectious diseases and maintain adequate levels of water hygiene.

Conflict of interest

The authors declare that they have no conflict of interest.

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