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Isolation and Characterization of Bacteriophages with Lytic Effect Against Waste Water Bacterial Pathogens

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ABSTRACT

Phage therapy is the therapeutic use of lytic bacteriophages to treat pathogenic bacterial infections especially multiple antibiotic-resistant bacteria infections. Our study aimed to isolate and purify different bacteriophages and investigate their activity against multidrug-resistant pathogenic bacteria. Sewage wastewater samples were collected from El-Shaheed City, Sohag, Egypt. All procedures for isolation and identification of bacteriophage and factors affecting on phage activity were performed at microbiological laboratory at Faculty of Science, Al-Azhar University. Our study showed that, from wastewater samples we isolated and identified three bacterial isolates **(***Pseudomonas aeruginosa*, *Salmonella sp.* and *Escherichia coli*)**,** mean inhibition zone by phage increased growth progressively and significantly with incubation periods from day 1 to the 6^{th} day for all tested bacteria, while inhibition zone increased in 1% of NaCl then decreased progressively to 9% NaCl. Different bacteriophages in our study were exposed to UV radiation at 254 nm in the range of 5–90 minutes. *Pseudomonas aeruginosa* phage was survival in UV exposure for 70 minutes, coliphage was survival for 75 minutes, while *Salmonella* sp. was survival for 80 minutes. The activity of phage against three bacterial isolates was increased progressively from pH 4 to pH 7 and then decreased activity to pH 10. Activity *Pseudomonas aeruginosa* and *Salmonella* sp. phage had thermal inactivation point, 90°C, whereas *Escherichia coli* phage at 80°C.

Condensed research regarding the isolated phages from different sites in Egypt, as well, incubation period and salinity percentage with more samples urged for significant results.

INTRODUCTION

Bacteriophages as a word is derived from the Greek word "phagein" means to eat and scientifically is defined as the viruses that specifically infect bacteria (Sieiro *et al*., 2020).

Bacteria are prokaryotic microorganisms that in their life processes evolve to turn into antibacterial resistant traits.

The advent of antibacterial-resistant strains in the world creates a grave challenge and cost to lives around the world, especially in most parts of developing countries (LaBauve and Wargo, 2012).

Bacteriophages (bacterial viruses) are the most predominant living organisms on earth exhibited on various environmental conditions (Clokie *et al*., 2011). Many literatures that studied the various forms of the discovered phages have revealed they are double-stranded DNA (dsDNA), tailed phages and belong to the order Caudovirales. The mentioned phages are characterized by the presence of a polyhedral head being most frequently icosahedral and tail that acts as a molecular machine to guide the virus recognizing its host and their tails as an injection structure where it injects nucleic acid into the bacterial cell wall and plasma membrane. Nonetheless, phages exhibit various forms due to their difference in virions, genomes and lifestyles (Cai *et al*., 2019).

Phages cannot replicate in eukaryotic cells or incorporate their DNA into the genome of such cells. They are highly specific in their bactericidal potential. They generally target a single bacterial species and some phages are even strain specific (Doore *et al*., 2019). This means that there is little or no effect on the natural macrobiotic of the patient animal (Rohde *et al*., 2018). This makes phages superior over many anti-bacterial agents. No reports about phages having harmful effects on eukaryotic cells. For instance, humans exposed to phages in the natural environment every day without adverse side effects, indicating their nontoxic nature. This is owed to their lack of tropism for mammals and their ability to penetrate tissues and barriers of eukaryotes to make them perfect candidates in therapeutic purposes (Huh *et al*., 2019). The restriction to infection and propagation in prokaryotic hosts renders human hosts safe from unintentional phage infection, although immunomodulatory phage-mammalian interactions should still be considered. Phages also can degrade bacteria present in a biofilm, mucous membrane and superlative wounds (Rohde *et al*., 2018; Huh *et al*., 2019; Sieiro *et al*., 2020). Ezenobi and Okpokwasili (2016) added a synergistic effect expected from phages and antibiotics. Dual phage antibiotic therapies could lead to a reduction in the emergence of antibiotic resistant strains.

Water and wastewater filtration systems usually contain pathogenic bacteria which must be removed to ensure safe potable water. High selectivity of bacteriophages to remove the potential pathogens e.g., *Pseudomonas aeruginosa* in drinking water was reported by Zhang *et al*. (2013). Biofilters and granular activated carbon (GAC) used in water treatment processes to improve water quality, remove micropollutants, and odor can provide a good environment for the survival and growth of bacterial pathogens because of their porosity. The contamination of such biofilters might be difficult to treat with chlorine disinfection. The use of phages that target specific pathogenic bacteria is a promising alternative to chemical disinfection (Zhang *et al*., 2013).

This study aimed to isolate phage from sewage sludge, identify its enteric bacterial host(s), and examine its potential use as a technology to remove harmful bacteria from recreational and potable water sources without causing harm to natural nonpathogenic bacterial assemblage.

MATERIALS AND METHODS

Source of Sewage Water Samples:

Isolates were picked from sewage water samples collected from the sewage treatment plant in El-Shaheed city and El-kawther city (Sohag governorate, Egypt).

Bacterial Isolation:

Successive dilutions of the collected samples from sewage water were prepared. 0.1 ml from each dilution was transferred on MacConkey agar medium plates, and then plates were incubated at 37° C for 24-48 hrs. The isolates were marked for purification on nutrient agar medium plate and then a single colony was selected and transferred onto slant surface of nutrient agar in a test tube; the tube

was incubated at 37°C for 48 hrs. and then stored at 4°C.

Microscopic Examination:

A single colony of each isolate was fixed on a clean slide to study Gram stain, under a light microscope (Atlas *et al*., 1995). Pure colonies were identified according to their Gram staining and another microscopically characteristic. A smear was prepared on a glass slide and Gram stained. The prepared smear was microscopically examined.

Biochemical Tests:

The suspected isolates were subjected to the biochemical tests as mentioned by methods reported by Macfadden (2000), the biochemical properties of bacterial isolates were performed as a confirmatory study for morphological identification to identify the bacterial isolates. In biochemical characters, we studied catalase, citrate, gas peroxide, gram stain, hydrogen sulfide, indole, oxidase and methyl red.

Bacteriophages Isolation and Identification:

The spot test was used for detection of phages in the collected sample. The spot test indicated that phages of identified bacterial isolates were found to be common in the collected sample.

To isolate bacteriophage, a liquid enrichment technique by Adams (1966) was used to isolate phages specific to bacterial isolates from the collected sewage water samples. Twenty ml of sewage water were incubated overnight with 40 ml of peptone water medium at 37° C. Five ml of chloroform were then added and the sample was shaken for 10 min, followed by centrifugation at 4000 rpm, for 10 min to remove bacteria. The supernatant was added to 3 ml of 24hrs old liquid culture of each bacterial isolate separately. After the multiplication of phages 24-30h at 30-33° C, bacteria were killed by shaking with 5 ml chloroform for 10 min, and then the sample was clarified by centrifugation at 4000 rpm. for 10 min. The supernatant was subjected to phage detection.

Detection of Bacteriophages (spot test):

Using Adams (1966) technique, a base layer of 10 ml of nutrient agar medium with 1.5% agar was poured in each petri dish (10 cm in diameter) and allowed to solidify. Three ml nutrient agar melted medium containing 0.7% agar was mixed with 300 µl of the indicator bacterial isolate was poured into each plate. The indicator bacteria were a liquid culture of 24hrs old of each bacterial isolate separately. The supernatant (phage lysate) was spotted with sterile micropipette on the upper layer after it had been solidified and then plates were incubated at 30-33° C for 24-48 h. A lysis of bacterial lawn was observed at the sites where drops had been applied. Each lysed clear zone was picked and transferred separately into eppendorf tube containing 1 ml of SM buffer (SM Buffer is a mixture of Sodium chloride, Magnesium sulphate and gelatin) (Maniatis *et al*., 1982). Two hundred µl of chloroform were added to each Eppendorf tube and then maintained at 4° C.

Purification of Phages:

The single plaque isolation technique was used to obtain pure phage isolates of each separately. The phages specific to bacterial isolate formed single plaques of different morphologies. Twenty morphologically different single plaques were selected and kept as pure phage isolates.

The High Titer Phage Suspensions:

The above agar double layer prepared according to Adams (1966) was used to prepare the titer. High titer phage suspensions as described by Hammad and Dora (1993) and Farahat (2016). 100 ml of high titer phage suspension were prepared for each phage isolate of bacterial isolate separately. Double layer plates were prepared as described above but the top layer contained a mixture of 3 ml of nutrient medium (0.7% agar), 300 µl of liquid culture of indicator bacteria and 20 µl of the diluted phage suspension. The plates were incubated at 30-33 °C for 24−48h. Plates that showed almost complete lysis were selected. 5 ml of SM buffer were added to the surface of each selected plate. The top agar layer of each plate was scraped off and combined in a flask together with the added SM, and then the occasional shaking was done for 30 min. Agar and bacterial debris was sediment by centrifugation at 4000 rpm for 30 min. The supernatant that containing the phages was transferred to another

sterilized flask, then 3 ml of chloroform were added and stored at 4° C. The titers of the prepared suspensions of the twenty phage isolates specific to indicator bacteria were ranged from 43 x 10^8 to 58 x 10^8 pfu/ml.

Effect of Certain Environmental Conditions on The Activity of Phage Against Different Bacterial Species:

Incubation Period:

Fifty ml of the nitrate broth medium were dispensed among conical flasks of 250 ml. Three flasks were used for each particular incubation period. The flasks were then sterilized, cooled, inoculated and incubated on a rotary shaker of 120 rpm at 38°C. Cultures were tested after 1, 2, 3, 4, 5 and 6 days of incubation for antibacterial activity.

Tolerance to NaCl Concentration:

The nitrate broth medium was supplemented with different concentrations of sodium chloride covering the range of 1- 9 %. Then, autoclaved and inoculated with microbial culture under study and incubated. At the end of the incubation period, all plates were tested visually for the presence of any obvious growth.

Tolerance to Temperature Degrees:

Tolerance to temperature degrees was tested using nitrate broth medium, from 10-100 ^{o}C. In all cases, the inhibition rate was recorded.

Determination of the Optimum Ph For Phage Infection:

The infectivity of the three phage isolates was studied at various pH levels (4-10). At each pH level tested, all phage isolates formed lysed spots.

Sensitivity to Ultraviolet Irradiation:

The sensitivity of the isolated phages of each bacterial isolate separately to UV radiation (at a wavelength of 254 nm) was studied. UV at different exposure times**.**

Electron Microscopy:

Five µl of high titer phage suspension was placed onto a sheet of parafilm. A farmavar 200 mesh coated grid was placed (farmer side down) on the drop of high titer phage suspension and allowed to absorb for approximately 10 min. The excess liquid was removed with a filter paper wick. The grid was placed on a drop of filtered 0.5% uranyl acetate PH 4.5 for 10 - 30 seconds (Stacey *et al*., 1984). The excess stain was removed with filter paper. The grid was air-dried, then examined at 50 kv in the transmission electron microscope (Joel, Model GEM 1010) in Assuit University - Assuit - Egypt.

Statistical Analysis:

Frequencies, percentages, and means were used, as appropriate, for descriptive analysis. Univariate and multivariate logistic regression analyses were performed to assess the significant predictors of three bacterial isolated and phage activity. All statistical analyses were conducted by SPSS software for Windows, release 18 (SPSS Inc., Chicago, IL, USA). A P-value<0.05 was considered significant.

RESULTS

Characterization and Identification of Bacterial Isolates: -

Three isolates were suspected to be *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella* sp., according to morphological characteristization and subjected to the related biochemical tests. The identified biochemical characters for *Pseudomonas aeruginosa* were positive catalase, citrate and oxidase test. The biochemical characters for *Escherichia coli* were positive catalase, gas peroxide, indole and methyl red test. Finally, biochemical characters for *Salmonella* sp. were positive catalase, hydrogen sulfide and methyl red test (Table 1).

Mean incubation periods affecting *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella* sp. growth were increased progressively and significantly from day 1 to the 6 days of incubation with a mean value increased from 0.03±0.001 - 0.466± 0.103, 0.830±0.125 to 1.322 ± 0.111and 2.645±0.211 to 4.371± 0.130, respectively. *Pseudomonas aeruginosa* significantly increased growth in 1% of NaCl then decreased to 0.088±0.02 in 9% NaCl. *Escherichia coli* significantly increased growth in 1% of NaCl then decreased to 0.636±0.118 in 9% NaCl. Finally, *Salmonella* sp. significantly increased growth in 1% of

NaCl then decreased to 2.525±0.338 in 9% NaCl (Tables 2&3).

Table 1: Biochemical tests for identified bacteria**.**

Test	*Pseudomonas aeruginosa*	*Escherichia coli*	*Salmonella* sp.
Catalase	+ ve	+ ve	+ ve
Citrate	+ ve	- ve	- ve
Gas	- ve	+ ve	- ve
Gram stain	- ve	- ve	- ve
H_2S	- ve	- ve	+ ve
Indole	- ve	+ ve	- ve
Oxidase	+ ve	- ve	- ve
Methylred	- ve	+ ve	+ ve

Table 2. Mean factors of incubation periods and NaCl conc. affecting *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella* sp. growth was measured as optical density at 623 nm.

	Pseudomonas aeruginosa							*Escherichia coli*							*Salmonella* sp.						
	M	SE	CV %	SD	Min	Max	*P Value*	M	SE	CV%	SD	Min	Max	*P Value*	M	SE	CV%	SD	Min	Max	*P Value*
	Incubation Period (days)							Incubation Period (days)							Incubation Period (days)						
1	0.031	0.001	11.174	0.003	0.024	0.035		0.830	0.125	45.281	0.375	0.220	1.450		2.645	0.211	24.034	0.635	1.700	3.650	
2	0.131	0.033	76.623	0.100	0.024	0.275		0.986	0.135	41.309	0.407	0.350	1.550		2.814	0.229	24.501	0.689	1.800	3.850	
3	0.309	0.079	77.135	0.238	0.110	0.860	<0.001	1.201	0.100	25.102	0.301	0.650	1.600	<0.001	3.088	0.207	20.165	0.622	2.000	3.950	<0.001
4	0.406	0.091	67.586	0.275	0.089	0.800		1.288	0.091	21.185	0.273	0.800	1.650		3.554	0.155	13.084	0.465	2.850	4.300	
5	0.452	0.102	67.643	0.306	0.129	0.950		1.287	0.087	20.415	0.262	0.900	1.700		3.833	0.183	14.362	0.550	3.000	4.600	
6	0.466	0.103	66.411	0.309	0.145	0.974		1.322	0.111	25.304	0.334	0.900	1.850		4.371	0.130	8.927	0.390	3.800	5.000	
	NaCl Conc.							NaCl Conc.							NaCl Conc.						
1%	0.564	0.161	70.252	0.396	0.033	0.974		1.633	0.055	8.364	0.136	1.450	1.850		4.225	0.207	12.039	0.508	3.650	5.000	
2%	0.549	0.144	64.371	0.353	0.031	0.886		1.503	0.061	9.970	0.149	1.250	1.700		3.975	0.236	14.577	0.579	3.200	4.800	
3%	0.424	0.108	62.709	0.265	0.024	0.653		1.381	0.084	14.955	0.206	1.010	1.600		3.741	0.254	16.665	0.623	3.010	4.650	
4%	0.304	0.080	64.532	0.196	0.035	0.503		1.238	0.090	17.805	0.220	0.850	1.400		3.525	0.263	18.291	0.644	2.800	4.450	
5%	0.210	0.056	66.008	0.138	0.028	0.352	<0.001	1.121	0.096	21.123	0.236	0.720	1.310	<0.001	3.383	0.255	18.473	0.625	2.750	4.300	<0.001
6%	0.181	0.047	63.646	0.115	0.029	0.289		1.061	0.082	19.010	0.201	0.710	1.220		3.308	0.242	17.942	0.593	2.700	4.220	
7%	0.151	0.036	59.319	0.089	0.032	0.242		0.973	0.074	18.648	0.181	0.710	1.180		3.003	0.307	25.113	0.754	2.100	4.120	
8%	0.223	0.129	141.418	0.316	0.025	0.860		0.825	0.103	30.757	0.253	0.460	1.000		2.775	0.326	28.823	0.799	1.900	4.000	
9%	0.088	0.02	56.683	0.050	0.024	0.145		0.636	0.118	45.594	0.290	0.220	0.900		2.525	0.338	32.843	0.829	1.700	3.800	

(**M**) Actual means; (**SE**) standard error; (**CV%**) coefficients of variation; (**SD**) standard deviations; (**Min**) Minimum; (**Mix**) maximum.

Table 3. Least squares mean NaCl concentrations and growth rate for *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella* sp.

Incubation Period (days)	*Pseudomonas aeruginosa*	*Escherichia coli*	*Salmonella* sp.
	LSM ± SE	LSM ± SE	LSM ± SE
1	0.031 ± 0.057 ^{b}	0.830 ± 0.026 ^{d}	2.645 ± 0.045 ^{f}
2	0.131 ± 0.057 ^{b}	0.986 ± 0.026 ^{c}	2. 814 ± 0.045^{e}
3	0.309 ± 0.057 ^{a}	1.201 ± 0.026 ^{b}	3.088 ± 0.045 ^{d}
4	0.406 ± 0.057 ^{a}	1.288 ± 0.026 ^{a}	3.554 ± 0.045 ^{c}
5	0.452 ± 0.057 ^{a}	1.287 ± 0.026 ^{a}	3.833 ± 0.045 ^{b}
6	0.466 ± 0.057 ^{a}	1.322 ± 0.026 ^{a}	4.371 ± 0.045 ^{a}
NaCl Conc.			
1%	0.564 ± 0.070 ^{a}	1.633 ± 0.032 ^{a}	4.225 ± 0.055 ^{a}
2%	0.549 ± 0.070 ^{a}	1.503 ± 0.032 ^{b}	3.975 ± 0.055 ^{b}
3%	0.424 ± 0.070 ^{ab}	1.381 ± 0.032 ^{c}	3.741 ± 0.055 ^{c}
4%	0.304 ± 0.070 ^{bc}	1.238 ± 0.032 ^{d}	3.525 ± 0.055 ^{d}
5%	0.210 ± 0.070 ^{bc}	1.121 ± 0.032 ^{e}	3.383 ± 0.055 ^{de}
6%	0.181 ± 0.070 ^{c}	1.061 ± 0.032 ^{ef}	3.308 ± 0.055 ^{e}
7%	0.151 ± 0.070 ^{c}	0.973 ± 0.032 ^{f}	3.003 ± 0.055 ^{f}
8%	0.223 ± 0.070 ^{bc}	0.825 ± 0.032 ^{g}	2.775 ± 0.055 ^{g}
9%	0.088 ± 0.070 ^{c}	0.636 ± 0.032 ^{h}	2.25 ± 0.055 ^{h}

(LSM) least-squares means**; (SE)** standard errors.

Phage Activity Against Different Bacterial Isolates:

The mean diameter of inhibition zone for three bacterial isolates were 0.748±0.05, 0.885±0.05, 0.917±0.060 in *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella* sp., respectively. All significantly differ with an increase in the mean value for *Salmonella* sp. (Table 4).

The activity of phage against three bacterial isolates was started at pH 4 and increased curvy with pH increased to high activity at pH7 and then the activity of bacteriophage decreased to pH 10 (Table 5).

Determination of Thermal Phage Inactivation:

Activity *Pseudomonas aeruginosa* and *Salmonella* sp. phage have thermal inactivation points at 90°C, whereas *Escherichia coli* phage at 80°C (Table 6).

Table 4. Phage activity against different bacterial isolates**.**

Species	M	SE	CV%	SD	Min	Max	*P Value*
***Salmonella* sp.**	0.917	0.060	17.476	0.160	0.750	1.200	**<0.001**
Escherichia coli	0.885	0.050	15.187	0.134	0.700	1.100	
Pseudomonas aeruginosa	0.748	0.050	17.846	0.133	0.550	0.950	

(**M**) Actual means; (**SE**) standard error; (**CV %**) coefficients of variation; (**SD**) standard deviations; (**Min**) Minimum; (**Mix**) maximum.

Table 5. Least squares mean of phage activity at different pH factors on bacterial isolates

Variable	LSM ± SE
***Salmonella* sp.**	0.917 ± 0.021 [a]
Escherichia coli	0.887 ± 0.021 [a]
Pseudomonas aeruginosa	0.748 ± 0.021 [b]
pH	
4	0.703 ± 0.033 [e]
5	0.836 ± 0.033 [cd]
6	0.983 ± 0.033 [ab]
7	**1.050 ± 0.033** [a]
8	0.900 ± 0.033 [bc]
9	0.783 ± 0.033 [de]
10	**0.696 ± 0.033** [e]

(**LSM**) The least-squares mean, (**SE**) standard errors

Table 6. Thermal points impact on *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella* sp. phage.

Temperature (° C)	*Salmonella* sp.	*Escherichia coli*	*Pseudomonas aeruginosa*
10	+	+	+
20	+	+	+
30	+	+	+
40	+	+	+
50	+	+	+
60	+	+	+
70	+	+	+
80	+	-	+
90	-	-	-
100	-	-	-

Application of UV Against Phage Activity:

Different bacteriophages in our study were exposed to UV radiation at 254 nm from range 5–90 minutes (Fig. 7). *Pseudomonas aeruginosa* phage was survival in UV exposure for 70 minutes, coliphage was survival in UV exposure for 75 minutes, while *Salmonella* sp. was survival in UV exposure for 80 minutes.

Table 7. Effect of UV radiation (at 254 nm) on bacteriophages

Strains	Exposure time (Minutes)																
	5	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
Pseudomonas aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
Escherichia coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Salmonella sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−

DISCUSSION

The coliphages has inhibited the normal growth of *E. coli* SBSWF27 during 30 hours of incubation at 37º C. Sewage treated with biological processes can be still unsafe and could be pathogenic consequently oxidative substances such as chlorine, UV, or ozonation are used to clarify any residual fecal pathogens (Sjahriani *et al*., 2021). Beyi & Leta (2019), revealed that their samples were obtained from different sites at Wollega university campus, *E. coli* was found in all water samples. *E. coli*, as most of the phages, depends on the availability of nutrients and energy for growth and survival. *E. coli* was reported to survive for days at > 30 º C temperature under aerobic condition for a year even under limited nutrition) (van Elsas *et al*., 2011).

According to microscopically examination, our study showed that, from sewage wastewater samples we isolated three bacterial isolates (*Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* and *Escherichia coli*), the biochemical characters of identified *Pseudomonas aeruginosa* were positive catalase, citrate and oxidase test. The biochemical characters for *Escherichia coli* were positive catalase, gas peroxide, indole and methyl red test. Finally, biochemical characters for *Salmonella* sp. were positive catalase, hydrogen sulfide and methyl red test. A similar study reported that *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* and *Escherichia coli* is ubiquitous Gram-negative and is the most common pathogenic bacteria in the world (Bell, 2002; Al-kafaween *et al*., 2019; Costa *et al*., 2019). The bacterial isolates were gram-negative rods in shape, non-spore-forming, with peritrichous flagella (Merchant and Packer, 1967).

The present study recorded that, mean incubation periods for *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella* sp. increased progressively and significantly from day 1 to the 6 days of incubation. All tested bacterial isolated were increased growth in 1% of NaCl then decreased in 9% NaCl. According to Khan *et al*. (2010) it was the first time for a study to detect *P. aeruginosa* in oceans and marine environment due to resistance to high salinity. *P. aeruginosa* was thought to tolerate river outlets and or freshwater or sewage. Another study by Deshwal &Kumar (2013) concluded that upon the isolation of different strains of *P. aeruginosa*, only 0 to 0.75% of NaCl were able to grow the bacteria while higher NaCl concentrations gradually reduced the growth. Incubation of *P. aeruginosa* in seawater for a long period of 14 years and adaptation of bacteria to high salinity was successful (Elabed *et al*., 2019). The study also revealed that higher NaCl concentration has a negative impact on all strains yet with longer incubation period biological replicates increased indicating exhaustion of the growth medium (maximum exhaustion was detected at 24 hr in acidic media) (Bushell *et al*. 2019).

The isolated phages formed circular single plaques of 1-3 mm in diameter and clear in appearance (Kiraly, *et al*., 1970; Elmaghraby *et al*., 2015).

The present study recorded that, *Pseudomonas aeruginosa* phage was survival in UV exposure for 70 minutes, *coliphage* was survival in UV exposure for 75 minutes, while *Salmonella* sp. was survival in UV exposure for 80 minutes. Other studies revealed to phages were found in environments with high UV irradiation and heat as the Sahara (Pachepsky *et al*., 2014) or hot springs (Percival *et al*., 2014) or in environments with various pH values and ionic strengths like in food (Kausar and Qamer, 2016), cheese factories (Bracciale *et al*., 2020), humans (Bach *et al*., 2005), soil (Carla *et al*., 2020), and sewage (Iliadis *et al*., 2018). Moreover, maintenance of phage populations usually requires the presence of the bacterial host, which is also influenced by environmental factors (Yang *et al.*, 2020).

In the present study, we reported that, *Pseudomonas aeruginosa* and *Salmonella* sp. phage have thermal inactivation point at 90°C, whereas coliphage at 80°C. The mean diameter of inhibition zone for three bacterial isolates are 0.748±0.05, 0.885±0.05, 0.917±0.060 in *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella* sp., respectively.

Bacteriophages are generally considered to be acid sensitive, but they differ strongly in their response to acidity. There are phages that are highly sensitive even to moderate pHs, but others which are quite resistant to even very low pHs (Keerthirathne *et al*., 2016).

Our study showed that, the activity of phage against three bacterial isolates were stared at pH 4 and increased curvy with pH increased to high activity at pH7 and then activity of bacteriophage decreased to pH 10. Similar study recorded that, as to a "phage acceptable" pH range, more sensitive phages survived only at pH 6.0 to 8.0 (eg PM2 phage) while others survived at pH 3.0 to 11.0 (Maal *et al*., 2015). Another study revealed to the propagation of numerous phage species is affected when pH is less than 4.5 despite the presence of ample bacterial cells. In human or animal systems, for instance, the application of oral phage may be influenced by stomach acidity that negatively disturbs the persistence of the phage and leads to loss of titer and treatment failure (O'Toole *et al*., 2015).

Bacteriophage isolates specific to *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella* sp. were negatively stained and examined by electron microscope. All phage isolates were found to be of oval head and small tail type. A similar result was Liu *et al*. (2020) revealed that isolated coliphage was from *Myoviridae* family of bacteriophages and the second lytic bacteriophage had an oval head measuring 98 × 35 nm and a small tail measuring 14 × 14 nm probably related to the *Podoviridae* family of bacteriophages.

Conclusion:

Sewage water sample were collected from sewage treatment plant of El-Shaheed city, Sohag, Egypt, and the isolated samples revealed the presence of *E. coli*, *Salmonella* sp., and *Pseudomonas aeruginosa*. Regarding salinity percentage, 2% NaCl revealed the highest growth rate in all species detected. pH of 7 was optimal for *Salmonella* sp. and *Pseudomonas aeruginosa* while pH of 6 was convenient for *E. coli*.

Authors' Contribution:

All authors are in agreement with the content of the manuscript and were involved in all steps of its preparation.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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