

New Approach to Use Phage Therapy against *Aeromonas hydrophila* Induced Motile *Aeromonas* Septicemia in Nile Tilapia

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Abstract

Infections with *Aeromonas hydrophila* is a progressive problem in aquaculture. The use of antibiotic such as Ciprofloxacin has contributed to the rapid and effective treatment of disease caused by this organism. However the fast-paced increase of resistance to the said antibiotics has posed problems and there is now a new approach to look for alternative methods to control this bacterial pathogen. Phage therapy comes in as a new method to respond to these growing problems. This study demonstrated the promising action of isolated bacteriophage ΦZH1 and ΦZH2 for therapy against Motile *Aeromonas* Septicemia in Nile tilapia caused by *Aeromonas hydrophila*.

Keywords: Phage therapy; *Aeromonas hydrophila*; Motile *Aeromonas* Septicemia; Fish; Nile tilapia

Introduction

Egyptian aquaculture has developed rapidly in recent years, where there are many problems facing fish, one of them is bacterial infection for fish, which constitutes a huge menace for aquaculture farming, leading to disastrous economic loss and health risks for the consumer [1].

Aeromonas hydrophila a gram negative, rod shaped enterobacterium and distributed widely in aquatic environments [2]. It is one of the most important agents of the outbreaks in fresh water fish. The main problem involving the use of antibiotics against *Aeromonas* infections is the development of resistance by these bacteria [3].

A bacteriophage is a virus that infects bacteria and can either instantly kill a bacterial cell or integrate its DNA into the host bacterial chromosome [4]. If the phage DNA is integrated into the host, the phage can then stay within the bacteria causing no harm. This pathway is called the lysogenic cycle. On the other hand, the phage can also cause eventual lysis and death of the host after it reproduces inside the host and escapes with numerous progeny through the lytic cycle [5]. Phages are effective against multidrug resistant pathogenic bacteria because the mechanisms by which they induce bacteriolysis differ completely from those antibiotics. Moreover, phages have self-limitation, meaning that the number of phages remain in very low level after killing the target bacteria [6].

The role of bacteriophages in the environment has been the subject of intense investigation over the past several years [7]. The development of techniques to study natural viral populations in situ has progressed tremendously. Various aspects of bacteriophage ecology in nature-including abundance, role in microbial mortality and water column trophodynamics, viral decay rates, repair mechanisms and lysogeny are gradually being understood [8]. Much of research has focused on using phages to control diseases caused by a variety of human pathogenic bacteria including *Salmonella* [9], *Listeria* [10] and *Campylobacter* [11] species. In addition, the current attempts to apply phages in the control of human pathogens, aquatic animal pathogens have also been investigated as a target for phage therapy.

A number of phages have been isolated for potential use in phage therapy against important aquatic animal pathogens such as

Aeromonas salmonicida in brook trout (*Oncorhynchus fontinalis*) [12], *Vibrio harveyi* in shrimp (*Penaeus monodon*) [13], *Pseudomonas plecoglossicida* in ayu (*Plecoglossus altivelis*) [14] and *Lactococcus garvieae* in yellowtail (*Seriola quinqueradiata*) [14].

The efficiency of an *Aeromonas hydrophila* bacteriophage isolated from ponds of Abbassa was compared to that of the antibiotic Ciprofloxacin for the treatment of "Motile *Aeromonas Septicemia*" (MAS) in *Oreochromis niloticus* [15]. Hence, this study aims to isolate and identify of *A. hydrophila* of lytic phages and efficiency of phages to control *A. hydrophila* in aquaria.

Material and Methods

Isolation of *Aeromonas hydrophila* bacteriophages

Bacteriophages were isolated from sewage samples by the specific enrichment method of Adams. The supernatant was filtered through a 0.45 µm pore size syringe filter and assayed for phage activity by double layer agar technique. The presence of phage in filtrate was detected by spot test and plaque assay methods as described by Eisenstark [16]. Phages were propagated and purified from single-plaque isolates according to Adams [17]. Plaques were distinguished by differences in plaque morphology, size and turbidity and were purified by successive single plaque isolation using the propagating host strain. Afterward, Phage suspensions of high titer lysates were prepared in two ways first Confluent plate lysates were prepared according to the method of Eisenstark [16].

Physical characterization of isolated bacteriophages

Effect of temperature on isolated phage: The effect of temperature

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on the viability of phage was studied by the method described by Clokie [18]. Phage suspension was incubated at 30, 40, 50, 60, 70, 80 and 90°C in water bath for 10 min. Phages survival was determined

Effect of irradiation by ultra violet light on the isolated bacteriophages: The effect of UV light on the viability of phages was studied by method described by Clokie [18]. UV sensitivity was determined by exposing 5 ml of phage lysate (4.8×10^{11} pfu/ml and 5.0×10^{11} pfu/ml) for phage ΦZH_1 and ΦZH_2 respectively (diluted 0.1 in saline solution) in an uncovered small Petri dish to UV-light at distance 20 cm from Cosmolux UVA, A1-11-40 W, PREHEAT- BIPIN, Mode In W-Germany, lamp was used as a UV source for the following times: 20, 40, 60, 80, 100 and 120 minutes. Phages survival was determined by plaque assay technique [17].

Biological Characteristics

Host range

The host ranges of the phages were determined by spot tests [16]. The adsorption experiments and single step growth curve of phages were carried out as described by Adams [17].

Effect of different MOI on bacterial growth

Multiplicity of infection (MOI) was defined as the ratio of virus particles to potential host cells and prepared according to Birge [19].

Morphological characteristics (Electron microscopy)

High stock titer of phages (4.8×10^{11} pfu/ml, 5.0×10^{11} pfu/ml) were negatively stained with 2% (w/v) aqueous uranyl acetate (pH 4.0) on a carbon-coated grid and examined by transmission electron microscopy JEOL (JEM-1400cx) at an accelerating voltage of 80 KV.

Effect of bacteriophages on mortality of Nile tilapia by *Aeromonas hydrophila* infections

Nile tilapia (*O. niloticus*) (weight range: 25-40 g) were obtained from ponds of Fish Research Center of Abbassa, Abo- Hammad, Sharkia. "Where these fishes were transferred alive to Microbiological laboratory, Faculty of Veterinary Medicine, Zagazig University. All fishes were kept in tanks (40 cm×70 cm×60 cm) with approximately 45 l de-chlorinated tap water. Acclimatized for 1 week prior to the experiment and fed with organic feeds. Four aquarium were used for the experiment and each aquarium contained 5 fishes. The aquaria were maintained at $28 \pm 1^\circ\text{C}$ with a pH of 7. Bacterial inoculum of *A. hydrophila* was prepared using a 24 hrs old culture of *A. hydrophila* inoculated in TSB. The inoculum was subjected to 10^{-7} , 10^{-8} and 10^{-9} dilutions. These were transferred to falcon tubes and were centrifuged for 30 min at $3,000 \times g$. After centrifugation, the supernatant was removed and 5 mL of normal saline solution (0.9%) was added. The lethal dose (LD) of *A. hydrophila* was determined by intraperitoneally injecting the fishes with 0.5 mL of doses [20]. The dose sufficient to cause death among the fishes within 72-96 h was taken as the optimum LD100 (lethal dose causing 100% mortality). Clinical signs of MAS such as skin lesions, hyperemia, rotting of caudal and dorsal fins, and hyperemia in fin bases were observed prior to the experimental treatment [21].

The mortality in the tanks was monitored after 15 days for each challenge. The concentration of bacteria and phage in the water tanks was monitored by inoculating the corresponding dilution in TSA plates to detect the bacteria, and using the double-layer agar plaque assay to determine the phage concentration [17].

Two groups only were injected with *A. hydrophila*. Group 1, which

contain water only. Group 2, which contain water and fishes served as the negative control. Group 3, which was used as positive control contain water and fishes were injected with *A. hydrophila* but was not treated with bacteriophages. Group 4, was also injected with *A. hydrophila* and was treated with bacteriophage. Administration of bacteriophage (Group 4) was done 24 hrs after injection of *A. hydrophila*.

Results

Morphology of plaques and phage(s)

Result in Table 1 and Figures 1 and 2 showed that two phenotypic plaques were appeared. One of them remarked as ΦZH_1 which measured 3.0 mm in diameter with turbid center (LTC), while other remarked as ΦZH_2 which measured 4.0 mm in diameter with clear center (LCC).

Morphology of isolated plaques under electron microscope

Five successive transfer plaques with clear area and center were selected to prepare height titer phage stocks (4.8×10^{11} pfu/ml) and (5.0×10^{11} pfu/ml). Each phage stock viewed under electron microscope

Phage No.	Appearance of plaques	Diameter of isolated phages (nm)	
		Head	Tail
ΦZH_1	LT	100	30
		100	30
ΦZH_2	LC	50	7.0

Table 1: Morphology of plaques and isolated phages under electron microscope after negatively staining.



Figure 1: Plaques morphology of *A. hydrophila* phage ΦZH_1 with turbid center on the TSA Plate.



Figure 2: Plaques morphology of *A. hydrophila* phage ΦZH_2 with clear center on the TSA Plate.

after staining by (1%) potassium phosphotungstate at pH 6.4. Results in Table 1 and Figures 3-5 showed that, two phages were appeared. Icosahedral heads of these phages (ϕZH_1 and ϕZH_2) measured 100 and 50 nm respectively. Also these phages had very short non-contractile tail measured 30 and 7 nm respectively. Phages (ϕZH_1 and ϕZH_2) adsorbed on cell wall and not have receptor on flagella (Figure 6). On the basis of phage morphology, the phages ϕZH_1 and ϕZH_2 belongs to the family Podoviridae [22].

Host range

The host range of isolated phages (ΦZH_1 and ΦZH_2) was determined against isolates of *Aeromonas* bacteria and 4 strains of non-*Aeromonas* bacteria. Results in Table 2 revealed that the isolated phages ΦZH_1 and ΦZH_2 was very specific to infect *Aeromonas* and does not have the ability to infect any isolates of non-*Aeromonas* bacteria.

Effect of thermal inactivation

The infectivity of both phages was highly sensitive to temperature above 40°C. Whereas both phages lost its infectivity by percentage reached to 88% and 50% for ΦZH_1 and ΦZH_2 respectively (Figure 7).

Effect of irradiation by ultraviolet light on the isolated phages

The exposure of 10^{11} pfu/ml purified phages suspensions to UV

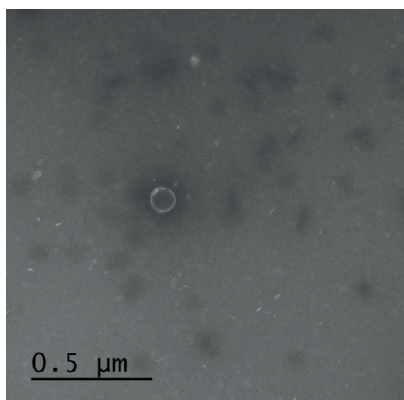


Figure 3: Electron micrograph of *A. hydrophila* phage ΦMH_1 . The purified bacteriophage preparation was negatively stained with (1%) potassium phosphotungstate (pH 6.4).

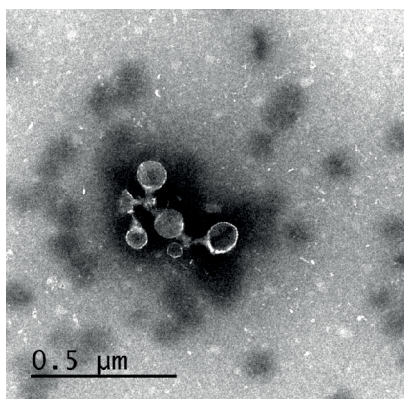


Figure 4: Electron micrograph of *A. hydrophila* phage aggregation of ΦMH_1 & ΦMH_2 . The purified bacteriophage preparation was negatively stained with (1%) potassium phosphotungstate (pH 6.4).

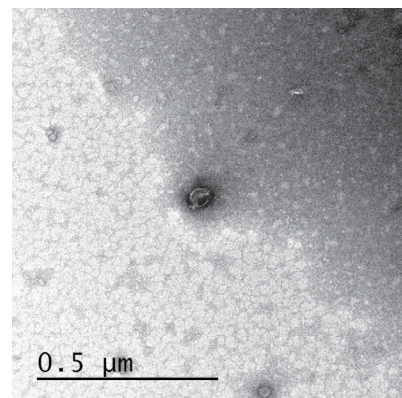


Figure 5: Electron micrograph of *A. hydrophila* phage ΦMH_2 . The purified bacteriophage preparation was negatively stained with (1%) potassium phosphotungstate (pH 6.4).

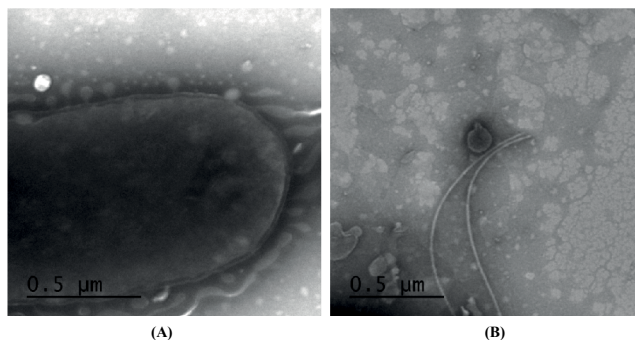


Figure 6: Electron micrograph of *A. hydrophila* both phage ΦMH_1 & ΦMH_2 adsorbed on cell wall (A) and not have receptor on *A. hydrophila* flagella (B).

	Hosts	Sources	Formation of lytic area by spot test
<i>Aeromonas hydrophila</i> bacteria	1		-
	2		+++
	3	Ponds of Aquaculture Research	-
	4	Center of Abbassa, Abo-Hammad, Sharkia	-
	5		-
	6		-
Non <i>Aeromonas hydrophila</i> bacteria	<i>Escherichia coli</i>	Central Laboratory of aquaculture Research Center of Abbassa, Abo-Hammad, Sharkia	-
	<i>Listeria meningitis</i>	Central Laboratory of aquaculture Research Center of Abbassa, abo-Hammad, Sharkia	-
	<i>Staphylococcus aureus</i>	Faculty of Science, zagazig University (Accession number KR270348)	-
	<i>P. aeruginosa</i> 62	Faculty of Pharmacy, Zagazig University (Gen bank Bio project 219845)	-

+/-, plaque /no plaque formation.

Table 2: Host range of *A. hydrophila* phages ΦZH_1 and ΦZH_2 .

irradiation (at high 20 cm) for different periods of time (0-120 min) illustrated by Figure 8 from these results, isolated phages (ΦZH_1 and ΦZH_2) are resistant to UV irradiation. Whereas the infectivity of this phage still active after exposure to UV (40w) for 120 min. ΦZH_1 lost

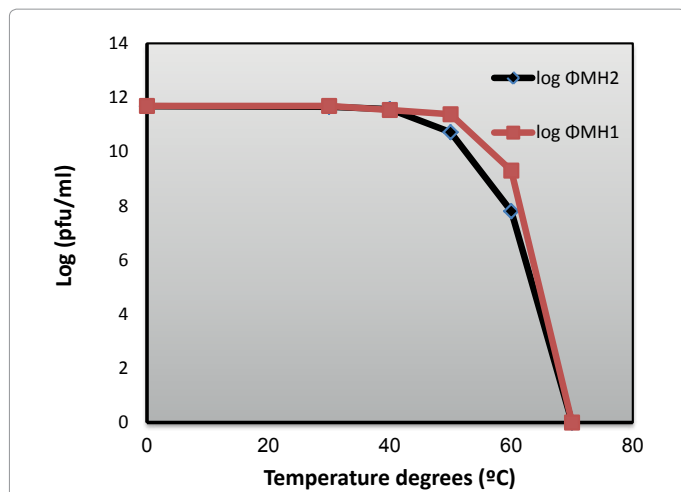


Figure 7: Effect of temperature on phage ΦZH_1 and ΦZH_2 . Phages was incubated at different temperature for 5 min and the phage infectivity was investigated.

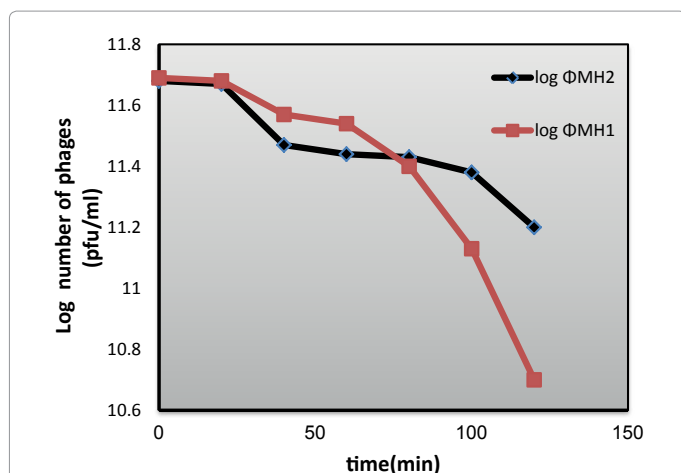


Figure 8: Effect of time exposure to UV irradiation on ΦZH_1 and ΦZH_2 phages.

50% of its infectivity after exposure to UV irradiation for 100 min. While, phage ΦZH_2 reached to this percentage after exposure to UV for 80 min.

Adsorption rates

The two phages in current studies ΦZH_1 and ΦZH_2 exhepted to different rates. The maximum adsorption and percentage of adsorption are presented in Table 3. Adsorption rate were fast since the maximum phages adsorbed reached 51% and 66.8% of phages ΦZH_1 and ΦZH_2 were adsorbed after 20 and 30 min respectively the adsorption constant (K) were $(2.7 \times 10^{-13} \text{ ml/min})$ and $(2.2 \times 10^{-13} \text{ ml/min})$ for ΦZH_1 and ΦZH_2 respectively as determined by the formula $K = 2.3 / (B) t \times \log (P_0/P)$, where P_0 = phage assay at zero time, P = phage not adsorbed at time t min, (B) = concentration of bacteria as number of cells/ml and K = velocity constant expressed as ml/min. Adsorption rate constant for both phages were similar.

One-step growth experiment

One step growth curve (Figure 9) shows that the latent period was

about 20 min, the rise period was 60 min, and the mean burst size was about 113 and 114 pfu per infected cell for ΦZH_1 and ΦZH_2 respectively.

Effect of different concentration of phages on growth of *A. hydrophila*

Each phage was used at MOI = 10, 1 and 0.1 over a time from 0-24 hrs. Data in Table 4 showed that, the highest reduction in bacterial count was observed when phage ΦZH_1 and ΦZH_2 added separately to *A. hydrophila* at M.O.I = 10 and incubated at 37°C for 12 hrs. On other hand, addition of phage ΦZH_1 or ΦZH_2 to *A. hydrophila* at M.O.I less than 10 (1.0 or 0.1) not gave efficient in reduction of bacterial growth.

The challenges to motile *Aeromonas* Septicemia (MAS) causing bacteria by both ΦZH_1 and ΦZH_2

Result in Table 5 showed that the addition of *A. hydrophila* to fishes in aquaria containing Nile water increases the mortality compared to it control. 2nd aquaria mortality of this treatment reached 68%. Addition of phage to such treatment reduced the mortality to 18% with reduction efficiency reached above 50%. Also phage reduced total count of bacteria from 4.8×10^{13} cfu/ml to 7.3×10^7 cfu/ml. on other hand, phages titer in fourth treatments increased above the initial titer of phages it changed from 8.1×10^9 to 8.1×10^{13} pfu/ml.

Discussion

Fish diseases are major problem for fish farming industry and among those bacterial infections are considered to be a major cause of mortality in fish [23]. These awaited drawbacks enforced the fish pathologists to seek for other alternatives; the use of natural

Incubation time (min)	ΦZH_1		ΦZH_2	
	pfu/ml	%	PFU/ML	Log
0	4.8×10^{11}	100	5.0×10^{11}	100
10	4.0×10^{11}	20	2.11×10^{11}	57.8
20	2.45×10^{11}	51	4.0×10^{11}	20
30	2.55×10^{11}	49	1.66×10^{11}	66.8
40	2.71×10^{11}	46	1.75×10^{11}	65
50	4.0×10^{11}	20	4.10×10^{11}	18
60	3.8×10^{11}	20	6.4×10^{11}	-
70	5.8×10^{11}	6	6.9×10^{11}	-

Table 3: Adsorption rate of *A. hydrophila* phages ΦZH_1 and ΦZH_2 in TS broth at 37°C MOI >1.0.

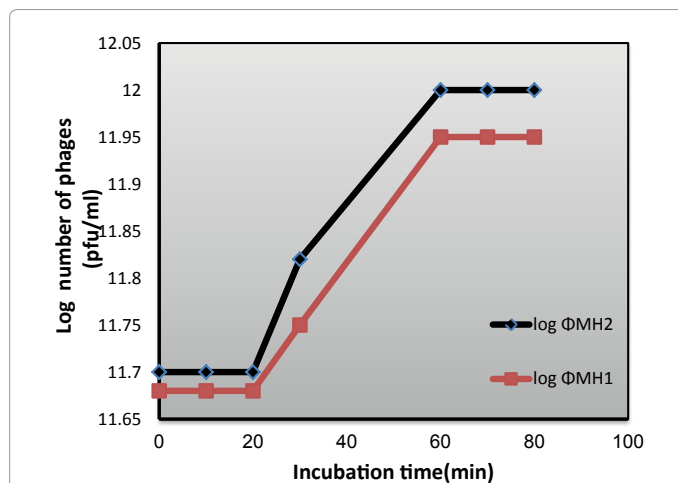


Figure 9: One step growth curve of *A. Hydrophila* phages ΦMH in TSB at 37°C. Initial titer of $\Phi ZH_1 = 4.8 \times 10^{11}$ (pfu/ml) and Initial titer of $\Phi ZH_2 = 5.0 \times 10^{11}$ (pfu/ml) and MOI <1.0.

Time	A. hydrophila		ΦZH_1						ΦZH_2					
			MOI=0.1		MOI = 1		MOI =10		MOI=0.1		MOI = 1		MOI=10	
	Bacterial count	OD	pfu	cfu	pfu	cfu	pfu	cfu	pfu	cfu	Pfu	cfu	pfu	cfu
6	1.8×10^{13}	0.324	4.1×10^{11}	1.9×10^{13}	5.5×10^{16}	2.1×10^{13}	7.3×10^{17}	1.7×10^{13}	4.5×10^{13}	1.8×10^{13}	2.8×10^{13}	2.2×10^{13}	3.8×10^{13}	2.2×10^{13}
12	2.5×10^{13}	0.450	4.0×10^{13}	2.3×10^{13}	6.1×10^{16}	2.4×10^{13}	6.8×10^{17}	3.1×10^{11}	4.0×10^{13}	4.6×10^{13}	2.1×10^{13}	3.8×10^{13}	9.5×10^{14}	2.1×10^9
18	3.6×10^{13}	0.648	3.8×10^{13}	3.1×10^{13}	6.8×10^{16}	2.1×10^{13}	5.4×10^{17}	3.4×10^{11}	3.9×10^{13}	4.1×10^{13}	1.7×10^{13}	3.4×10^{13}	4.9×10^{16}	1.8×10^9
24	4.2×10^{13}	0.756	3.0×10^{13}	2.8×10^{13}	7.1×10^{13}	2.0×10^{13}	3.8×10^{17}	3.3×10^{11}	3.2×10^{13}	4.2×10^{13}	1.8×10^{13}	2.9×10^{13}	6.0×10^{16}	1.1×10^9

Table 4: Effect of incubation of several different multiplicity of infection (M.O.I) on growth curve of the host *A. hydrophila*.

Treatment	Mortality%	Total bacterial count after 72 hrs.	Phages titer after 72 hrs.
Nile water	0.00	5.2×10^{11}	0.00
Nile water + fishes	5.00	3.9×10^{11}	0.00
Nile water + fishes + <i>A. hydrophila</i> (3.73×10^9 cfu/ml)	68.00	4.8×10^{13}	0.00
Nile water + fishes + <i>A. hydrophila</i> + phages (8.1×10^9 pfu/ml) (MOI=2.1)	18.00	7.3×10^7	8.1×10^{13}

Table 5: The challenges to Motile *Aeromonas* Septicemia (MAS) causing bacteria by both ΦZH_1 and ΦZH_2 .

immunostimulants in fish culture for the prevention of diseases was a promising new development and could solve the problems of massive antibiotic use. Natural immunostimulants were biocompatible, biodegradable and safe for both the environment and human health. Moreover, they possess an added nutritional value [24].

Aeromonas hydrophila was described as the dominant infectious agent of 'fish-bacterial-septicemia' in freshwater cultured finfish all over the world [25]. *A. hydrophila* was also associated with EUS, which was a major problem in different countries [26]. The observed clinical signs in the examined fish suffering from motile *Aeromonas* Septicemia (MAS) were previously reported by Samal [25]. They reported that septicemia, ascitis, erosion, ulceration, detachment of scale, exophthalmia and muscular necrosis were the most predominant clinical signs of MAS in Nile tilapia.

In this study, the results showed that two bacteriophages ΦZH_1 and ΦZH_2 possess infection on its specific host *A. hydrophila*, isolated from Nile water. According to the electron micrograph; the two phages were characterized as podoviruses. The dimensions of the isolated podoviruses were similar or semi-similar to each other and also resembled those which were previously isolated for *A. hydrophila* [27]. *A. hydrophila* phages ΦZH_1 and ΦZH_2 infected some *A. hydrophila* strains, but none of other genera or species tested. These results are in accordance with those obtained by Mitchell Sc [28].

Temperature is a crucial factor for bacteriophage survivability [29]. The results in showed that ΦZH_1 and ΦZH_2 phages were thermos table, between a temperature ranges of 30-60°C where it still remained active after 10 min exposure at 60°C. Interestingly, ΦZH_1 and ΦZH_2 phages survived at 37°C, with no significant loss in phage particle number, which is a very important parameter for phages considered for therapeutic application.

Phages examined here were tolerant to UV irradiation with distinct rate of inactivation where ΦZH_1 and ΦZH_2 lost 50% of their infectivity after exposure time reach to 100 and 80 min respectively. These result are in accordance with those obtain by Ramanandan [29]. This finding indicated that phages are suitable to use in field experiment where their infectivity not affected by UV in solar reached to water in aquarium.

The data obtained in one- step growth experiment were comparable with data presented by Cheng [30]. These authors conducted a similar growth experiment with *Aeromonas* species. It as reported by those authors that the latent periods of phage DH1 were 90 min which was much longer than *Aeromonas hydrophila* phages Aeh1 and Aeh2 [28]

on other hand in our studies the latent period of phages ΦZH_1 and ΦZH_2 were 20 min. the average burst size of phage DH1 were about 125 PFU/cell, which was also bigger than Aeh1 and Aeh2 [28], but the latent period of phages ΦZH_1 and ΦZH_2 were 113 and 114 pfu per infected cell. The isolated phages (ΦZH_1 and ΦZH_2) administered via injection was found to be effective in treating fish infected with *Aeromonas hydrophila* shown through the significant decrease in number of *A. hydrophila* found in the water of treated fish. Where our results showed that the addition of phages ΦZH_1 and ΦZH_2 (M.O.I=2.1) to Nile water in aquaria. Which was inoculated by *A. hydrophila* (3.37×10^9 CFU/ml) reduced the percentage of mortality from 68% to 18% after treatment for 15 days. Also total number of bacteria in polluted aquarium changed from 4.18×10^{13} cfu/ml to 7.5×10^7 Cfu/ml after three days of treatments. The efficiency of isolated phages reduction of *A. hydrophila* in Nile water more than that founded by Donn Cruz-Papa [15].

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