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Isolation and Molecular Characterization of Some Marine *Aeromonas* phages: Protective Effects for Nile Tilapia Infected with *Aeromonas hydrophila*

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The goal of the current study is to evaluate the potential of marine phages for therapy of motile *Aeromonas* septicemia caused by *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*). To achieve this goal, four different *Aeromonas* phages namely AP1, AP2, AP3 and AP4 were isolated from seawater samples and tested for their potentiality as antibacterial agents against culture community in aquaculture water using RAPD-PCR. Results showed significant variation in their antibacterial effect. Characterization of the isolated phages based on restriction digestion using *EcoRI, Bam HI, HindIII* and *Eco RV* restriction enzymes and RAPD-PCR proved that the phages were not identical. Plackett Burman experimental design was applied for optimization of culture conditions to improve the antibacterial activity of the most promising AP2 phage against *A. hydrophila*. Results achieved 94% elimination of *A. hydrophila* comparing to phage infectivity under basal conditions. Invivo efficiency of AP2 against *A. hydrophila* invading the aquaria of Nile tilapia was investigated. Elimination of *A. hydrophila* in the rearing water was detected after 24h. Moreover, morphological and histological examination of the tested liver and gills tissues of Nile tilapia proved the promising effect of AP2 for therapy of motile *Aeromonas* Septicemia induces by *A. hydrophila.*

Keywords: Phages; *A. hydrophila*; phage therapy, Nile tilapia.

Aeromonas sp. is attributed to many diseases by producing cytotoxins, hemolysins and resistance to many antibiotics[1]. The most serious bacterial pathogen *Aeromonas hydrophila* in fish is responsible for many pathological conditions, such as, septicemia, tail rot and epizootic ulcerative syndrome [2].

Control of infection in Aquaculture was dependent on the use of chemical compounds, which resulted in the arising of antibiotic resistance. Therefore there is an urgent need to have alternative tools for pathogen control in aquaculture[3].

Phages are distributed in nature with increased numbers than their hosts. They can exist in aquatic system, wastewater, animal gut and springs [4]. They have been estimated to kill 20-40% of marine bacteria every day[5]. They are encouraged to be used in therapeutic applications due to their specific bactericidal capability.

In recent years, natural antimicrobial agents using lytic bacteriophages is a promising new alternative therapy to face the growing bacterial resistance to antibiotics [6,7], which has caused problems in different fields and aquaculture. Different studies concerned the use of *Aeromonas* phages in treatment of bacterial infection [8,9].

Different factors affect the success of phage frightening against the target pathogenic

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bacteria such as susceptibility to target bacteria, environmental conditions (temperature, pH, target bacteria /bacteriophages ratio, the time and mode of treatment[10].

Therefore the present study was planned to isolate and characterize different marine *Aeromonas* phages in addition to studying the potential antibacterial of the most potent phage against *A. hydrophila* under the optimized culture conditions as an alternative to the traditional chemotherapeutic agents.

MATERIALS AND METHODS

Samples

Sea water samples were collected from different sites along Mattrouh seashores using sterile screw capped bottles, transferred to the lab and stored at 4 ºC.

Media

The following media (g/l) were used throughout the present work (g/l): Nutrient agar: Peptone, 5; yeast extract, 3; beef extract, 1 and agar, 20. Luria Bertani (LB): Peptone, 10; yeast extract, 5. They were supplied from LAB M, England. The pH of each medium was adjusted to 7.0 ± 0.2 before autoclaving at 121°C for 15 min.

Isolation and purification of marine *Aeromonas* phages

Isolation, purification and determination of bacteriophage titer was carried out as previously described[11]. First, filtration of sea water samples was done using membrane filter (0.45 µm), followed by serial dilution of the filtrate. Diluted filtrates (0.5 mL) and 1 mL of the host *A. hydrophila* (in early log phase) were both added on 3 mL soft agar, the mixture was left to solidify. Plates were checked after 24 h of incubation at 30 ºC for plaque formation. In order to gain homogenous plaques, each plaque was collected, and used for repeated steps of *A. hydrophila* infection using double-layer method [12].

Antibacterial activity of the isolated phages against bacterial community structure using Random Amplified Polymorphic DNA (RAPD-PCR)

The isolated marine *Aeromonas* phages (AP1, AP2, AP3 and AP4) at 4.1×10^6 $PFUmL^{-1}$ were added separately to Erlenmeyer flasks containing 100 mL of aquaculture water collected from El-Mex fish farm. Another set without phage addition was kept as control (C). All sets were incubated at 30 °C for 24 h. After incubation, samples were filtered using 0.22 µm pore-size filters. The bacterial cells retained on the membranes were suspended in 2 mL of TE buffer (10 mM Tris HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0) and centrifuged[13]. In all cases, the extraction of DNA was carried out for further differentiation among different treatments compared to the control (un treated sample) using RAPD-PCR. PCR amplification was carried out by using a PCR Thermocycler (T100™ Thermal Cycler BIO RAD) in the subsequent setting: 1 round of 95°C for 5 min; 35 cycles of 95°C for 30s, 30°C for 30s, and 72°C for 1 min. The following primers were used:

A: GAATTTTTTAGGAGGACACTATGAGTG;
B: AGAGTTTGATCMTGGCTCAG;
C: TACGGYACCTTGTTACGACTT;
D: GAGTCGTATAAGATGAATAAGGGGGAATG;
E: ATCATATGTCTACATCCCTGAAGTAC;
F: TAGAGCTCTTACAGTCCCTTCGCTTGC

Molecular characterization of the isolated phages using RAPD-PCR

DNA of the isolated *Aeromonas* phages was extracted using (thermo) kit. The differentiation among phages was studied using RAPD-PCR, based on the occurrence or lack of an exacting RAPD band[14]. PCR amplification was carried out by using a PCR Thermocycler (T100™ Thermal Cycler BIO RAD) in the subsequent setting: 1 round of 95°C for 5 min; 35 cycles of 95°C for 30s, 30°C for 30s, and 72°C for 1 min using the following primers:

A: GAGTCGTATAAGATGAATAAGGGGGAATG;
B: TAGGCCTGTAAGCTTTTGTCTTGCACAATAAGT;
C: GAATTTTTTAGGAGGACACTATGAGTG;
D: AGAGTTTGATCMTGGCTCAG

RELP analysis

RELP were take place by restriction digestion analysis for the purified DNA isolated from phage isolates. The digestion reaction was performed using fifty units of *EcoRI, Bam HI, HindIII* and *Eco RV* (promega, Southampton, UK), the products of the reactions were analyzed by agarose gel (1%) electrophoresis[15].

Host specificity of the isolated phages

Different bacterial strains (Table 1) kindly, provided by Marine Microbiology Department, NIOF, Alexandria, Egypy were tested for their specificity to the isolated phages. Briefly, host cells

were grown to log phase and plated on nutrient agar plates. Different purified phage stocks were separately spotted onto the plates, and the plates were incubated overnight at 30 °C. Production of clear or turbid plaques was considered as positive record[16].

Table 1. Host specificity of the isolated *Aeromonas* phages

Host	AP1	AP2	AP3	AP4
Staphylococcus aureus	+	+	-	+
Streptococcus epidermidis	-	-	+	-
Vibrio parahaemolyticus	+	+	-	-
Vibrio alginolyticus	-	+	-	-
Escherichia coli	+	+	+	-
Enterococcus faecalis	-	-	-	+
Pseudomonas aeruginosa	-	+	-	-
Vibrio damsela	-	+	-	+

Optimization of culture conditions for improving the antibacterial activity against A. hydrophila using Plackett-Burman experimental design.

Effect of different factors on infectivity of AP2 to *A.hydrophila* was investigated by applying Plackett- Burman experimental design[17]. In this experiment, seven factors were screened in 8 combinations according to the Plackett Burman design matrix (Table 3). Increase of the original component level is shown by the (+) sign, while decrease of the original component level is shown by (-) sign. The main effect of each factor was determined using the following equation:

$$Exi = (Mi+ – Mi-) / N$$

Where Exi is the variable main effect, and Mi+ and Mi- are optical density (OD at 600 nm) in the trials, where the independent variables were present in high and low concentrations, respectively, and N is the number of trials divided by two. Microsoft Excel was used to calculate statistical t-values for equal unpaired samples and significance of the tested variables. From main effect results, an optimized conditions were predicted which will give maximum inhibition of bacterial growth, followed by verification step to confirm the accuracy of the optimized medium.

Electron microscopy examination

Specimen was negatively stained with sodium tungstate (2%) in bidistilled water at pH 6-7.5. and dropped onto a carbon coated grid. The excess liquid was removed with filter paper after 1 min. Five µl of dye solution were added and after 1 min, the grid was dried. The examination of grids were carried out and electron micrographs were taken with Transmission Electron Microscope (TEM) (JEOL 100 CX) operating at 80 kv.

In vivo efficiency of AP2 phage against *A. hydrophila*

Invivo efficiency was carried out using a modified method[8]. Nile tilapia (*O. niloticus*) (weight range: 5-9 g) were used in the study. All fishes were kept in tanks (47 cm x 33 cm x 30 cm) with approximately 5 L de-chlorinated tap water (10 fish per tank), acclimatized for 1 weak prior to the experiment and fed with organic feeds. The aquaria were maintained at 28 ± 1 °C with a pH of 7. Bacterial inoculum of *A. hydrophila* was prepared using a 24-h-old culture of *A. hydrophila* inoculated in LB. Four aquaria (T1-T4) were prepared, T1,T2 and T3 were infected with $28x10^4$ CFU ml^1 of *A. hydrophila*. On the initial day, AP2 was added to T2, T3 with the ratio of 1:1, 1:2 (AP2: *A. hydrophila*), respectively, while the first tank (T1) was kept without phage inoculation and T4 was kept as control. In all cases, the total bacterial viable count (TVC) and count of *Aeromonas* spp. were monitored daily during 72 h with platting of 100 µl of fish culture water on nutrient agar and m-Aeromonas agar medium with ampicillin selective supplement (SRO136), respectively and incubating at 37°C for 24 h. The experiment was carried out in duplicate and the results were taken as average ± SD. Two apparently healthy fish were collected from each tank for histological examination. Any dead fish was daily recorded and removed.

Histological examination

After fish dissection, the liver and gills were removed, thoroughly washed with a physiological saline (0.9% NaCl) solution and blotted on filter paper then buffered formalin 10%. The fixed specimens were processed using a conventional paraffin embedding technique. This was followed by staining 5 mm thick sections of the prepared paraffin blocks using Hematoxylin and

Eosin (HE) for light microscopic examination[18].

RESULTS AND DISCUSSION

Natural antibacterial agents are recommended due to their biocompatibility and safety for environment and human health. In the present study, phages specific to *A. hydrophila* were isolated from sea water samples using soft-agar overlay method. Among the isolated phages AP1, AP2, AP3, and AP4 representing different size and plaque morphology were selected for further study.

Screening for the antibacterial activity of the isolated phages against bacterial community in aquaculture water

In order to study the antibacterial impact of the isolated phages (AP1, AP2, AP3 and AP4) on the bacterial community in the collected aquaculture water from El-Mex fish farm, DNA extraction & RAPD profiles of the bacterial community was compared before and after addition of phages. Data indicated that all phage isolates showed a principal DNA with size larger than 10 kb. Concentration of DNA in the treated samples was 68 µg/µl, 369 µg/µl, 656 µg/µl and 454 µg/µl for treatment with Ap1, AP2, AP3 and AP4, respectively compared to 1937 µg/µl for the control (without phage addition), this variation was according to the effect of phage on the bacterial cells. Results of RAPD (Fig. 1) indicated that the addition of phages altered the bacterial ribotype diversity represented as difference in the pattern of bands, which indicated that the isolated phages are promising candidate as antibacterial agent in Aquaculture. In accordance with the current investigation, Pereira et al. (2011)[13] reported that *Aeromonas salmonicida* phage showed moderated effect on the structure of bacterial community after addition to aquaculture water.

Characterization of the isolated Aeromonas phages

Differentiation of phage DNA by RAPD-PCR

RAPD-PCR was used to detect differences among the isolated phages. As shown in Fig. 2, the RAPD-PCR profile of the isolated phages using different primers was varied which indicate that the AP1, AP2, AP3 and AP4 are not identical. RAPD was documented as quick typing and differentiation of phage isolates[19, 20].

Restriction Fragment Length Polymorphism (RFLP)

In this experiment a PCR reaction was followed by RFLP analysis with *EcoRI, Bam HI, HindIII* and *Eco RV.* According to (RFLP) pattern, phage isolates were classified. The results (Fig. 3) revealed that none of the tested PCR products showed a change in size after applying *HindIII* and *Bam* HI restriction enzyme; this means that there is no site for this enzyme within the tested fragments. Further digestion of PCR products with the other restriction enzymes lead to presence of variation between phage isolates. Data indicated that there are different positions for restriction digestion with these enzyme and the developed RFLP patterns of phage isolates are varied.

Cluster analysis

Tree of the isolated phages was carried out based on RAPD and RFLP showing different similarity levels and relationships between the four

Table 2. Growth factors affecting antibacterial activity of AP2 against *A. hydrophila* and their levels in the Plackett-Burman experiment

Factor	Symbol	Levels		
		-1	0	1
Peptone (g/l)	P	5	10	15
Yeast (g/l)	Y	2.5	5	7.5
Inoculum's size of host bacteria (ml)	ISb	0.5	1	1.5
Inoculum's size (ml) of phage	ISp	0.5	1	1.5
Sea water concentration (%)	SW	50	100	150
pH	pH	6	7	8
Temperature (°C)	T	25	30	37

isolates; it was obvious that AP2 phage was not identical to the other phages (Fig. 4).

Host specificity

Host specificity of the isolated phages was studied against eight bacterial strains (Table 1) using the spot test. Results indicated variation in host specificity, AP2 exhibited broad spectra of host specificity as it was able to infect all the tested pathogens except for *S. epidermidis* and *E. faecalis*. Consequently AP2 was choosen to complete the study. Host specificity is often associated with the differences in tail fiber proteins [21]. Another factor is conservative structure nature of phage receptors on the outer membrane of bacterial cell [22]. Another study reported that some marine phages are specific and lyse only the original host bacterium[23]. El-Araby et al. (2016)[8] studied the phage host specificity of two isolated marine *Aeromonas* phages namely θZH1 and θZH2 and stated that among the tested bacterial pathogens, the phages were specific to infect *Aeromonas* and do not have the ability to infect the other bacteria.

Optimization of culture conditions for improving the antibacterial activity of AP2 against *A.hydrophila*

Physicochemical parameters are important factors affecting survival and infectivity of bacteriophages[24].Thus the aim of this experiment is to recognize the most important factors affecting the infectivity of *Aeromonas* phage using Plackett-Burman experimental design. The screened factors and their levels were presented in Table 2. Results in Table 3 showed varied results of the antibacterial activity of AP2 against *A. hydrophila* (expressed as elimination of bacterial growth) during the period of study and 7 hours were the most suitable time for higher antibacterial activity. The main effect of the tested variables on the infectivity of AP2 and

Table 3. The applied Plakett-Burman experimental design for the seven culture variables with its antibacterial activity (expressed as bacterial growth (O.D.) at 600 nm)

	Factors symbol								Response (O.D. at 600 nm) at different time intervals (h)							
Trials	P	Y	SW	pH	Isb	ISp	T	0	1	2	3	4	5	6	7	8
1	-1	-1	-1	1	1	1	-1	0.020	0.030	0.045	0.060	0.160	0.600	0.210	0.230	0.280
2	1	-1	-1	-1	-1	1	1	0.020	0.105	0.190	0.350	0.500	0.490	0.910	0.790	0.800
3	-1	1	-1	-1	1	-1	1	0.020	1.180	0.010	0.100	0.120	0.670	0.030	0.010	0.010
4	1	1	-1	1	-1	-1	-1	0.020	0.160	0.320	0.390	0.420	0.620	0.440	0.260	0.460
5	-1	-1	1	1	-1	-1	1	0.020	0.170	0.320	0.490	0.510	0.590	0.540	0.140	0.160
6	1	-1	1	-1	1	-1	-1	0.020	0.032	0.040	0.280	0.230	0.560	0.390	0.010	0.010
7	-1	1	1	-1	-1	1	-1	0.020	0.490	1.870	1.850	1.840	2.230	1.490	1.530	1.520
8	1	1	1	1	1	1	1	0.020	0.710	1.830	1.360	1.270	3.050	2.110	1.610	1.700
9	0	0	0	0	0	0	0	0.020	0.042	0.030	0.200	0.560	0.800	0.920	1.70	0.450
Main effect	0.19	0.56	-0.215	0.94	0.5	-0.025	0.13									
t-value	0.25	1.24	-0.43	2.8	1.07	-0.05	0.26									

Table 4. Mean count of total bacteria and *Aeromonas* spp. in rearing water of *O.niloticus*

	TVC x10^4				*Aeromonas* spp. x10^4			
	Zero time	24h	48h	72h	zero time	24h	48h	72h
T1	28±1.27^{d}	4000±2.22^{a}	400±2.6.67^{b}	280±1.333^{c}	7±0.33^{d}	25±1.56^{c}	48±2.40^{b}	90±6.43^{a}
T2	28±2.33^{a}	2±0.11^{d}	7±0.47^{c}	13.6±0.65^{b}	7±0.58^{a}	2.5±0.16^{b}	1.8±0.09^{bc}	1.2±0.09^{c}
T3	28±1.56^{a}	0.2±0.01^{b}	24±1.60^{a}	24±1.14^{a}	7±0.39^{a}	2.5±0.16^{b}	1.25±0.06^{c}	1.19±0.09^{c}
T4	17 ± 0.78^{a}	30± 0.15^{b}	34±0.16^{b}	44 ± 0.19^{c}	5 ± 0.20^{a}	11±0.25^{b}	19±0.37^{b}	26±0.46^{c}

a, b, c, d indicate significant difference ($p< 0.05$).

their corresponding t-values were illustrated in Table 3. The current study indicated that increased concentrations of peptone and yeast extract exhibited positive effect on bacterial inactivation. It was reported that bacteria are more susceptible to phage infection in nutrient-rich conditions [25-28].

pH and temperature finding of the current study showed positive effect of AP2 in reduction of *A. hydrophial* as pH and temperature can interfere with phage attachment, thus preventing phages to infect the host. Infectivity of some bacteriophages was sensitive to pH values lower than five and more than ten[29]. Langlet et al. (2007)[30] reported that virus exhibited stability at wide range of pH. 37 °C was the most suitable temperature for phage infectivity which is in agreement with El-Araby et al. (2016)[8] showing that ÖZH1 and ÖZH2 *Aeromonas* phages survived better at 37°C and Taj et al. (2014)[28] who confirmed that 37°C was ideal temperature for infectivity for T4 bacteriophage. The negative effect of sea water concentration in the current study is coincide with a study by Fennema (1996)[31]who reported the denaturation of phage proteins at high sea water concentrations. Silva (2005)[32] showed that salt concentration affected infectivity of *V. vulnificus* and *V. parahaemolyticus* phages. Negative effect of phage inoculum was also detected, which is coincide with a study by Ly-Chatain (2014)[10]who stated that active biocontrol relies on the addition of a small amount of phages,

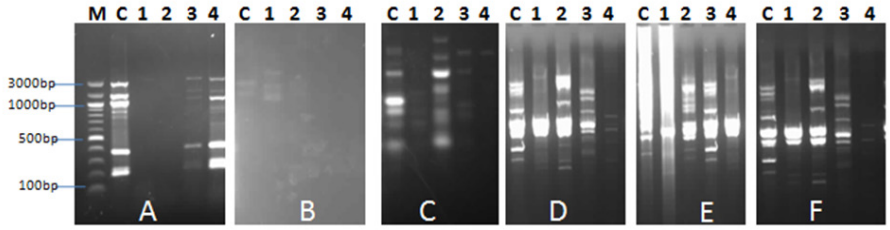

Fig. 1. RAPD PCR fragments using the primers: A, B, C, D, E. and F. after addition of AP1, AP2, AP3 and AP4 phages (Lanes 1-4) to bacterial community of the aquaculture system.. M: molecular weight marker, C: water samples (without addition of phages)

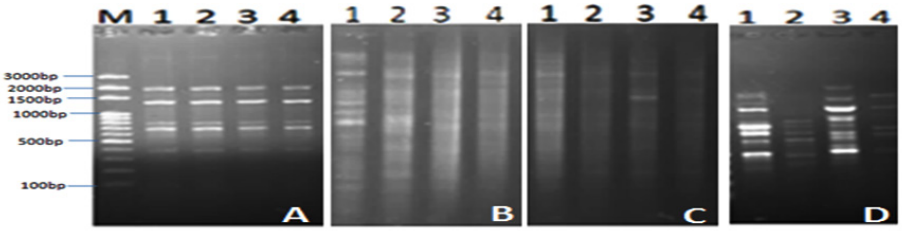

Fig. 2. Agarose gel (1 %) electrophoresis of RAPD products from AP1, AP2, AP3 and AP4 template DNA with the primers: A, B, C and D. Lanes 1-4 represent (AP1-AP4) phages. M is molecular weight marker

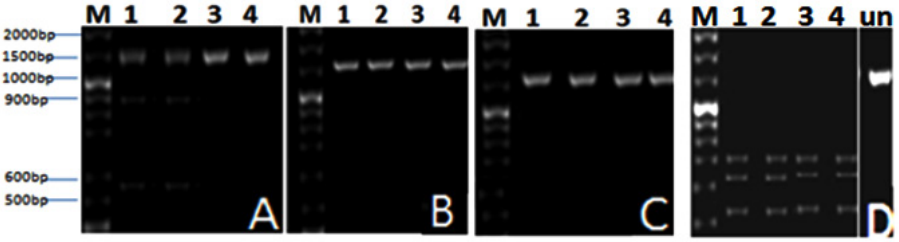

Fig. 3. Agarose gel (1 %) electrophoresis for DNA of *Aeromonas* phages AP1, AP2, AP3 and AP4 indicated by Lanes (1-4), cut with: A: *EcoRI,* B: *Bam HI,*C: *HindIII* and D: *Eco RV* restriction enzymes. M is molecular weight marker, (Un) is uncut PCR product

where elimination of bacteria, in this case, supposes the replication of phages over several generations.

The predicted optimum conditions were: Peptone, 15; yeast extract, 7.5; sea water concentration, 50%; bacterial inoculum size, 1.5; inoculum size of phage, 0.5; pH, 8 and incubated at 37 °C. According to values predicted using t-test (Table 3), pH and yeast extract concentration were highly significant variables.

A verification tests were performed to assure the validation of Plackett-Burman experimental design using the predicted optimized media against the basal condition. Results (Fig. 5) showed the increase in the infectivity of AP2 in terms of bacterial growth elimination by about 94% comparing to the phage infectivity under basal conditions. The effect of AP2 grown under the optimized conditions on *A. hydrophila* was monitored by TEM. As shown a rupture of *A. hydrophila* cell was observed after phage addition (Fig. 6A) compared to the control *A. hydrophila* (Absence of AP2) (Fig. 6B). Fig. 6 C showing the morphology of AP2 infecting *A. hydrophila*. Elimination *Aeromonas hydrophila* by action of *Aeromonas* phages was previously reported [8, 33].

In vivo* efficiency of AP2 in elimination of *A. hydrophila

In vivo efficiency of AP4 as antibacterial

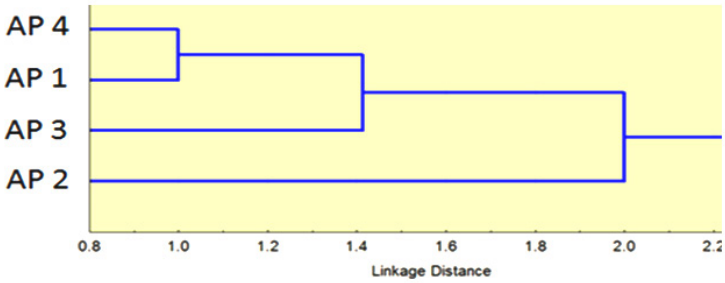

Fig. 4. Cluster analysis tree (Neighbors) based on RAPD and RFLP

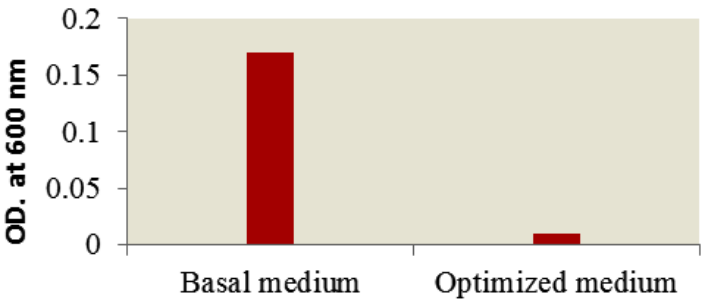

Fig. 5. Effect of optimized culture conditions versus basal medium on the antibacterial activity of AP2

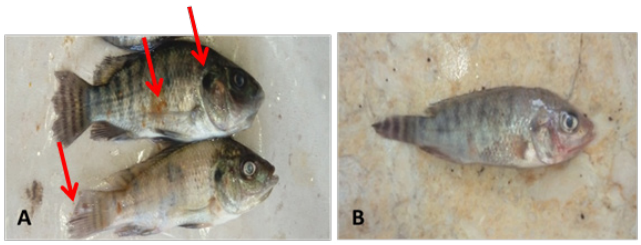

Fig. 6. TEM showing AP2 mediated lysis of *A. hydrophila* (A); *A. hydrophila* cell before lysis (B) and AP2 phage (C)

agent against *A. hydrophila* in aquaculture was investigated using different ratios of AP2: *A. hydrophila* (1:2, 1:1). As shown in Table 4, significant elimination ($P<0.05$) of TVC from $28x10^4±2.33$ to $2x10^4±0.11$ in rearing water of *O.niloticus* post 24h of AP2 addition was observed using 1:2 concentration of AP2: *A. hydrophila* (T2 treatment). Similarly, the antibacterial effect of AP2 against *Aeromonas* started 24h post treatment and prolonged until 72 h with gradual decrease in *Aeromonas* counts to reach 1.2±0.09 after 72 h compared to 90±6.43 CFU/ml in the infected aquaria. Also the mortality reached 40% during the treatment period. Protective effect of *Aeromonas* phages against *A. hydrophila* causing motile aeromonad septicemia was reported in different studies [8, 19, 34].

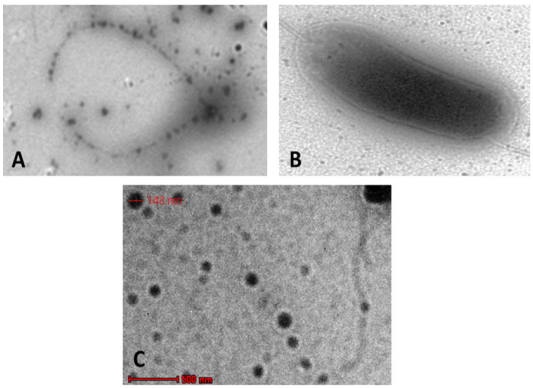

Fig. 7. Morphological symptoms: (A): infected *O.niloticus* with *A. hydrophila* showing darkness in skin with fin rot in the caudal fin, black spot on the operculum (shown by red arrows); (B) healthy *O. niloticus*

Morphological symptoms

Morphology of *O.niloticus* and infection signs were observed during 15 days. As shown in Fig. 7A, darkness in skin with fin rot in the caudal fin, black spot on the operculum were observed in the infected fish (shown by red arrows), while these signs were disappeared in the healthy fish (Fig. 7B). Similar signs were reported and attributed to infection with *A. hydrophila* [35, 36].

Liver histopathological alterations in *O. niloticus*

Histological sections of the liver (control) of *O. niloticus* (Fig. 8A) showed a prominent central vein, with cords of parenchyma cells (hepatocytes) leading to it. Blood vessels appear between the hepatic cells and surrounded by a thin layer of smooth muscle fibers. Nuclei usually centrally located with one prominent central nucleolus. On the other hand, it was noted that in liver of the untreated *O. niloticus*, small vacuoles of different sizes are scattered in the cytoplasm of some hepatocytes. The infected fish showed disruption of the normal hepatic cord-like pattern, a great reduction in size and number of hepatocytes. The hepatocytes show swelling with foamy cytoplasm and smaller nuclei and a prominent

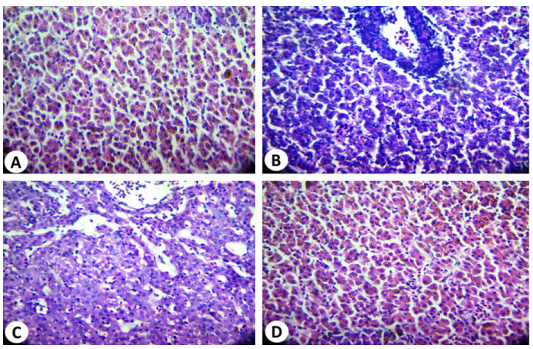

Fig. 8. Histopathological alterations of *O.niloticus* liver: (A): Normal liver tissue (control); (B): infected liver tissue showing disruption of the normal hepatic cord-like pattern, a great reduction in number and size of hepatocytes, (C): *O.niloticus* liver tissue treated with 1:1 (AP2 : *A. hydrophila*) and (D) *O.niloticus* liver tissue treated with 1:2 (AP2 : *A. hydrophila*) (D) (H&E: X, 400)

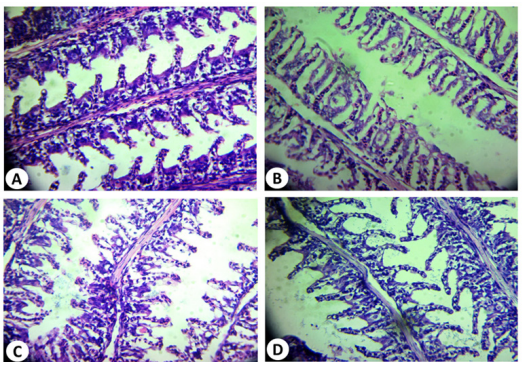

Fig. 9. Histopathological alterations of *O. niloticus* gills tissue: (A) normal gills tissue; (B): infected gills tissue showed separation of the epithelial lining of the secondary lamellae from its basement membrane; (C): *O.niloticus* gills tissue treated with 1:1 (AP2 : *A. hydrophila*) and (D) *O.niloticus* gills tissue treated with 1:2 (AP2 : *A. hydrophila*) (H&E: X, 400)

nucleolus which pushed aside. Most blood vessels were empty with red blood cells suffering from necrosis and haemolysis (Fig. 8B). The degree of degeneration is more prominent in case of *O. niloticus* treated with 1:1 (AP2: A. *hydrophia*) (Fig. 8 C). At *O. niloticus*, the hepatocytes had not great difference as compared to the control. However, it was noticed that nuclear degeneration was apparent and cytoplasm appeared more vacuolated than the control as shown in Figure 8D. Similar results have been shown for septicemia caused by *A. hydrophila* [36-38].

Gill histopathological alterations in *O. niloticus*

The structure of gills in the control fish was similar to that reported in previous study[39]. Two specialized epithelia with distinct blood compartments could be distinguished in a frontal section (Fig. 9A), the primary lamellae devoted mainly to ionic regulation and the secondary lamellae responsible for gas transfer[39]. The respiratory lamellae are lined by squamous epithelial layer one cell thick. In addition, there are a number of pillars, chloride and mucous cells. It is important to mention that; hyperplasia was observed in the epithelial cells of the primary lamellae in most control gills examined.

The infected fish gills showed separation of the epithelial lining of the secondary lamellae from its basement membrane as shown in Fig. 9B. Hyperplasia and hypertrophy of the epithelium of the primary lamellae and fusion of the adjacent secondary lamellae are shown in treated fish with 1:1 (AP4: *A. hydrophila*) (Fig. 9C). The adjacent respiratory lamellae were shortened due to curling or twisting and fused together making one side of the gill filament "solid" structure with no respiratory surface. The gills in case of 1:2 (AP2: *A. hydrophila***)** treatment may recover its shape of primary and secondary lamellae as shown in Fig. 9 D. These results are in accordance with previous reports[40,41].

CONCLUSIONS

The current study suggested the use of marine *Aeromonas* phages as an efficient and economical tool to control *Aeromonas* spp. The marine *Aeromonas* phage AP2 showed broad range of host specificity, which improves its potential as antibacterial agent. AP2 provided protective effects for Nile tilapia directed to motile *Aeromonas* Septicemia.

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