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ORIGINAL ARTICLE

Isolation and Characterization of Lytic Bacteriophages Infecting Fish Pathogen *Vibrio* Sp. Strains

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ABSTRACT
In this study, bacteria were isolated from infected organs of sea bass (Dicentrarhus labrax) and identified as Vibrio sp. according to 16S rRNA analysis. Four bacteriophages were isolated from seawater samples collected from the coast of Mersin province in Turkey. Vibrio sp. strains were used as hosts for bacteriophage isolation. Bacteriophages inhibit the growth of their hosts; Vibrio sp. K12, Vibrio sp. K11, Vibrio sp. L8, Vibrio sp. L5 which are potential bacterial pathogen of sea bass. Bacteriophages were characterized by Restriction Analysis (RA) and Transmission Electron Microscopy (TEM). Restriction Analysis showed that genomes of bacteriophages LP8, KP12, LP5 and KP11 are double stranded DNAs and the sizes of these genomes are 73, 55, 44 and 23 kb respectively. Only three out of phage were visualized by TEM. Electron microscopy studies revealed that bacteriophages LP5 and LP8 have an icosahedral head with a contractile tail and belonged to family Myoviridae. Bacteriophage KP11 shows binary symmetry, and belongs to family Siphoviridae with a long noncontractile tail. Experiments were carried out with three phages which were characterized by TEM. One-step growth experiments showed that the phages have different latent periods (30-40-50 min) and burst sizes (58-123-141) plaque-forming units (PFU) per infected cell at their host strains.
Keywords: *Dicentrarchus labrax, Vibrio* sp., lytic bacteriophages, *Siphoviridae*, *Myoviridae*.

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INTRODUCTION

Aquaculture is one of the fastest growing food production sectors worldwide. According to FAO, 2015 global aquaculture production has grown at an average rate of 5.9% per year and has reached to 76,6 million tonnes (http://www.fao.org/fishery/statistics/en Accessed February 2019). In addition to, aquaculture production has an important role in economic development. The reason of water-borne epidemics are viruses, bacteria, oomycetes, fungi, helmints and protists, nevertheless bacterial diseases are the main problems and leading to economic losses in the aquaculture industry [1-2-3]. Vibriosis is a well-known bacterial disease and widely responsible for mortality in aquaculture industry worldwide [4]. *Vibrio* sp. can cause vibriosis in fresh and salt-water fish which is economic important, such as sea bass *(Dicentrarchus labrax* L., 1758) [5-6]. Also, wide range of vibrios; *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus* have been recognized as causal agents of vibriosis [7]. Vibrios belong to the *Vibrionaceae* family, *Gammaproteobacteria*, are gram negative, usually motile rods, have a facultative fermentative metabolism, and common in marine and estuarine ecosystems [8]. Antibiotics have been used to control of vibriosis in aquaculture [9-10]. However, antibiotics may be ineffective against increasing antibiotic resistance. The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications [11]. Another problem is the presence of residual antibiotics incommercialized of aquaculture products and has led to allergy and toxicity in humans [12-13]. Due to disadvantages that arise from the consumption of antibiotics, alternative strategies are required for both minimizing the

hazardous effects of bacterial infection and prevention of diseases in aquaculture [14-15]. Bacteriophages are the most abundant living organisms in the World [16-17]. For a long time, there has been therapeutic use of phages against bacterial infections as an alternative to antibiotics [18-19]. New approaches acquired for the treatment of bacterial infections through occurrence of bacterial strains resistant to antibiotics. Phage therapy is not a new treatment but has a long history and is an important method to control bacterial infections [20]. Phage theraphy in aquaculture *Vibrio* sp control has also been studied quite intensively for 10 years [9-21-22]. Several reports described the isolation of phages of luminescent vibrios, including lysogenic ones and more recently also lytic phages from aquatic environments [23-24]. Here we present our work on the isolation and characterization of three selected bacteriophages against *Vibrio* sp. strains that were isolated from sea bass (*Dicentrarhus labrax*) and consider their potential as biocontrol agents in this fish species.

MATERIALS AND METHODS

Bacterial strains, sample collection and growth media

The bacterial strains used in host range experiment were purchased from ATCC collection. The host bacteria *Vibrio* sp. isolated from sea bass (*Dicentrarhus labrax*) collected at Delta (coordinate) near Mersin province. Fish samples were obtained from local equipped fishing boats in the area. Samples were immediately taken into a thermal bag that contain ice to prevent damage to the tissues. They were directly transferred to the laboratory, in aseptic conditions. Fish samples dissected to separate organs (kidney, liver) and each organ was homogenized individually. Homogenates were placed into 250 mL Tryptic Soy Broth (TSB, Merck, Germany) with 1.5% sodium chloride (NaCl) and incubated at 25°C overnight. Serial dilution of the sample (10^{-2} to 10^{-4}) streaked on Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS, Merck, Germany) and incubated at 25°C for 24 hours [25]. Selected colonies of different morphological types were isolated and sub-cultured to obtain a pure culture. All bacterial strains were stored in TSB containing 20% glycerol at -80°C until further use.

Identification of bacteria by 16S rRNA analyses

Bacterial genomic DNA was extracted using the Genomic DNA Kit (NanoBiz), following the manufacturer's recommended protocol. 16S rRNA gene was amplified by universal primers. PCR was carried out in a 50 µL reaction mixture, which contained genomic DNA template, 1X Taq buffer (Applied Biosystems, USA), 0,2 mM deoxynucleoside triphosphates (dNTPs, Fermentas, USA), primers (0,3 µM each), $MgCl_2$ (1,5 mM), 1,5 U Taq polymerase (Applied Biosystems, USA) and water. PCR amplification was performed with initial denaturation for 5 min at 95°C, followed by 35 cycles of 45 s at 95°C, 1 min at 51°C, and 2 min at 72°C and final extension at 72°C for 7 min. The 16S rRNA was generated with ABI 3130XL Genetic Analyzer (PE Applied Biosystems, USA). The amplified PCR products were cleaned using a Macherey-Nagel NucleoSpin Extract II kit (Macherey Nagel, Germany). Then, sequences were aligned using nucleotid BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence data were determined with software MEGA6. The phylogenetic tree was designated using bootstrap tests performed with 500 replicates and using the minimum evolution tree method [26].

Bacteriophage isolation, purification and enrichment

Sea water samples were collected from the coastal waters (top 30 cm) of Province, Country. Samples were centrifuged at 7000 rpm for 15 min [27]. Supernatants were filtered through 0.22 µm filter (Milipore, Merck, Germany). Filtrates were added to TSB (1.5% NaCl) before inoculated with log phase culture of host bacteria that isolated from sea bass. The phage and host were mixed and incubated with shaking (150 rpm) at 25°C for 24 h, then centrifuged (10,000×g, 10 min) (Hettich, Germany). Supernatant was used as phage suspension and presence of phage were determined by double agar overlay method [28]. 100 µL of phage suspension added to overnight culture of the bacterial hosts and incubated for 15 min at room temperature. Molten top agar (0.7% Agar, 4% TSB, 1.5% NaCl) was added to phage–bacteria mixture and mixed gently by rolling between the palms then poured onto Tryptic Soy Agar with 1.5% NaCl. Plates were incubated at 25°C overnight and inspected for zones clearing. One single plaque were picked up and replated three times to ensure pure phage isolates. Phage isolates were stored in SM buffer with 1% chloroform at 4°C. The phage titres were determined by the double agar layer method. The number of virus particle was counted and expressed as plaque forming units/mL (pfu/mL). The purified phage lysates were stained by 4–6-diamidino-2-phenylindole (DAPI) to determine the type of nucleic acid. Samples were examined with an Olympus BX51 (Japan) epifluorescence microscope [29].

Bacteriophage DNA extraction

High titer phage stocks and DNAs were prepared by using the methods described by Sambrook *et al*. [30]. DNA samples were extracted with GeneJET Gel Extraction and Cleanup Micro Kit (Thermo Fisher Scientific,

USA) by following manufacturer's recommended protocol (Carlson, 2005). The amount of phage DNA was determined by Nanodrop spectrophotometer (CapitalBio Nano, China).

Restriction enzyme analysis of bacteriophage DNA

DNAs were digested with restriction enzyme HindIII (Thermo Fisher Scientific, USA) following the manufacturer's recommended protocol. DNA fragments were separated by electrophoresis in 0.7% agarose gel. The molecular sizes of DNA fragments were calculated with DNA size software.

Transmission Electron Microscopy (TEM)

Bacteriophages were visualized by TEM to determine virus morphology. 10 µL of concentrated bacteriophage suspension (minimum 10^9 PFU/mL) in SM buffer were mixed with 100 µL of 2.5% glutaraldehyde buffer and incubated for 5 min at room temperature. Then, 20 µL of the sample were mounted on pioloform coated grids folllowed by the addition of 0.5% uranyl acetate. Samples were visualized under Transmission Electron Microscopy (Jeol, Japan) [32].

Phage host range determination

Determination of the host range was performed by spot test [33]. Each phage isolates was evaluated against different bacterial hosts. Bacterial strains used in host range experiment were purchased from ATCC collection and *Vibrio* sp. that isolated from sea bass.

Phage adsorption assays

A 10 ml of exponentially grown host culture were mixed with püre phage lysate at an multiplicity of infection (MOI) of 0.001 and were incubate at 25ºC. Aliquots of mixture were taken initially and every 10 minutes of incubation and chloroform (final volume 1%) was then added. Adsorbed phages were subsequently separated from nonadsorbed phages by centrifugation at 12.000 g for 5 min. The supernatants were filtered with 0.22 µm membrane (Milipore, Merck, Germany). Filtrats were plated in different dilutions together with an indicator strain to determine the titer. The plates were incubated at 25ºC and checked for plaques. As a control, phage lysates without bacteria were tested no-adsorption standard for measurements [34-35]. Three independent assays were done.

One step growth assay

One-step growth experiment was performed according to the procedure described by Mateus *et al*. [36] with some modifications. 10 µl of the each phage lysate (10^6 PFU/mL) was mixed with 10 mL *Vibrio* sp. culture (0.8 OD at 600 nm) to MOI= 0.001 and incubated at 25ºC. Host-phage mixture was centrifuged at 12.000 g for 5 min to remove the nonadsorbed phages, the pellet was washed and resuspended in 10 mL of TSB at 25ºC and then were diluted. Samples were collected every 10 minutes and phages titrated to determine the enhance in phage titres.

Prophage detection in the host bacterium Vibrio sp.

Mitomycin C is the standard chemical used to induce the lytic life cycle of temperate phages [37-38]. 50 µl of an overnight culture of *Vibrio* sp. and a the mixture of bacteriophages and their hosts *Vibrio* sp. were incubated in a water bath for 30 min at 25ºC. Mitomycin C (Sigma, USA) were added to a final concentration of 0.1–0.5 µg/ml. The samples were incubated overnight and centrifuged at 10000 g for 10 min at 4ºC. The supernatant were filtered through a 0.45 µm of pore size membrane filter. Then supernatant was controlled for the presence of phages.

RESULTS AND DISCUSSION

Identification of bacteria

Vibrios have emerged as a problem of marine fish and until now many species have been described as fish pathogens [25]. In this study, *Vibrio* sp. strains were isolated from the kidney and liver of sea bass. The bacterial strains produced yellow, viscose, round shaped colonies on TCBS medium after 24 h incubation at 25°C. Colonies that were isolated from liver were smaller than kidney isolates and showed black pigments on TCBS medium after 48 h incubation at 25°C. 4 bacterial strains were selected according to high growth parameters. Comparative ribosomal DNA gene sequence analysis indicated that the 4 bacterial strains (K11, K12, L5, L8) were in the same group as *Vibrio* genus (Figure 1). The strains had 96-97% sequence similarities with pathogen serotypes *Vibrio anguillarum* (Table 1). Species will be referred to as *Vibrio* sp. in this study.

Bacteriophage studies

Lately the phage therapy has received attention due to an increase in the currency of antibiotic resistant strains. A number of studies have been conducted that the efficacy of phages in treating different infections [36-39-40-41]. In this study, phages isolated by using *Vibrio* sp. strains as a host organisms. Double agar overlay plaque assay results showed that visible zones of lysis or plaques have been observed in four *Vibrio* sp. strains. Lytic bacteriophages were isolated, purified and named as LP5, LP8 and KP11, KP12. Bacteriophage strains showed different plaque morphology. Bacteriophages LP5 and LP8 formed small

plaques with a diameter varying between 0.5-1.0 mm. Bacteriophage KP11 and KP12 formed small, pinpoint, turbid plaques after about 24 h of incubation at 25°C. Only three of the bacteriophages were visualized and their morphology was determined by TEM analysis. Bacteriophage KP12 could not be observed, despite several attempts. Three phages were assigned to the order *Caudovirales* that includes the three families of tailed phages [42]. Electron microscopy studies revealed that all bacteriophages shows a binary symmetry; LP8 and LP5 have an icosahedral head with a contractile tails with tail fiber and KP11 have a head and with long noncontractil tail. According to criteria of head, tail, and genetic material; KP11 with noncontractile tail belonged to family *Siphoviridae* and LP5, LP8 with isometric head and contractile tail belonged to family *Myoviridae* (Figure 2, 3, 4) [43]. This finding is consistent with previous studies suggesting that water-isolated bacteriophages, and especially vibrio phages, belong to the families *Siphoviridae* and *Myoviridae* [39-44-45].

In our study, TEM examination of the three phage isolates showed that they were morphologically distinct and appeared to be similar to isolated phages of *Vibrio anguillarum* previously found [40]. According to Ackermann, capsid and tail size of tailed phages range from 30 to 160 nm and 1 to 800 nm, respectively [42]. Thiyagarajan et al. [46] isolated three phages that had an icosahedral head of 60–115 nm and a tail of 130–329 nm. In another study, *Vibrio harveyi* phage had an icosahedral head, approximately 60 nm diameter and non-contractile tail approximately 100 nm[23]. Higuera et al. [40] determined that the ALMED phage which is a *Myoviridae* family member had a long contractile tail and head of 50 nm. Our bacteriophages LP5, LP8 (*Myoviridae*) and KP11 (*Siphoviridae*) had 55-66-54 nm capsid sizes and 44-73-302 nm tail sizes, respectively.

In the marine environment most phages have the dsDNA genome, belonging mainly to *Caudovirales* [47]. In our study, restriction analyses showed that the type of phages nucleic acid was dsDNA. Bacteriophage DNAs were digested with HindIII restriction endonuclease according to the manufacturer's recommendations. According to agarose gel results, molecular sizes of DNA fragments were determined with DNA size software (Figure 5). Results showed that genome sizes of bacteriophages LP8, LP5, KP12 and KP11 are 73, 44, 55 and 23 kb, respectively. At the same time, phage particles were stained with DAPI and double-stranded DNA appeared as tiny bright blue fluorescent dots on the epifluorescence microscope (Figure 6). A summary of the genome sizes and morphological properties of the bacteriophages were presented in Table 2. There are many reports of isolation of Vibrio bacteriophages from coastal and marine environments [19-32-45-48]. Genome sizes of vibriophages have determined in many studies [45-46-49-50].

Higuera et al. [40] characterized six *Vibrio anguillarum* phages, according to the restriction patterns, the genome sizes of these vibriophages were 47-48 kb.

In another study, the results of the PFGE studies showed that 11 vibriophage genomes were different sizes among 11 kb to 244 kb [45]. Thiyagarajan et al. [46] reported that the molecular weights of the four vibriophages was estimated to be between 58-107 kb. Comeau et al. [44] characterized *V. parahaemolyticus* phages from different environments and found that the genome sizes of these vibriophages ranged from 45-106 kb. According to another study, *Vibrio harveyi* phage had 83 kb genome size that belonged to *Siphoviridae* family [23]. Shivu et al. [24] showed that genome size of six vibriophages ranged from 44-94 kb. Yu et al. [41] determined that the molecular weights of 5 phage, belonged to *Siphoviridae* family, ranging from 30 to 48 kb. It has been reported that vibriophages genome sizes have a wide range [24].

Prophage detection in the host bacterium after phage addition Cells with temperate phages usually result in the release of the phage after inducing it by mitomycin C. The experiment of mitomycin C showed that phages presented lytic cycles with no evidence of lysogeny induction [51-52]. In our study, no plaques were detected in the supernatant of cultures of *Vibrio* sp. or bacteria-phage mixture after treatment with mitomycin.

Staphylococcus aureus (ATCC 25953), *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* subsp. *spizizenii* (ATCC 6633) strains and *Vibrio fischeri* (ATCC 7744) were used for host range determinations of three *Vibrio* sp. bacteriophages. Three phages tested in this study, KP11 and LP5, LP8 showed lytic activity to their original indicator strains (100 %). Bacteriophage KP11 and LP5 showed lytic activity to only original indicator strains. Also bacteriophage LP8 showed clear zones with original indicator strain and *Pseudomonas aeruginosa* and (ATCC 9027). Phage LP8 infected *Pseudomonas aeruginosa* presented low efficiency of 53.21 % and *Vibrio sp*. L5 presented efficiency (82.27 %). The phage KP11 and LP5 infected presenting *Vibrio* sp. K11, *Vibrio* sp. L5 an efficacy of 100 %. The phage LP8 infected *Vibrio* sp. L8, *Vibrio* sp. L5 and *Pseudomonas aeruginosa* presenting an efficacy of 100 %, 82.27 % and 53,21% respectively. *Staphylococcus aureus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* subsp. *spizizenii* and *V. fischeri*

were not sensitive to KP11 and LP5 phages.The lytic activity of the phages for the bacteria are shown in Table 3.

Phage adsorption assays with KP11 showed that approximately 55% of the phage particles adsorb to *Vibrio* sp. K11 after 30 min and %67 adsorbed after 80 min. LP5 phage particles were adsorbed 62% after 50 minutes and about 83% after 70 min. 43% of LP8 phage particles were adsorbed to their host *Vibrio* sp. L8 in 40 min. and 89% of particles were adsorbed after 80 min. (Figure 7).

Lysis time and burst size were estimated from one-step growth experiment. KP11 showed an a latent period of 40 min, followed by the rise period of 20 min and burst size of 123 PFU/host cell. A latent period of 30 min and burst size of 141 PFU/host cell were calculated for LP8. The LP5 phage is characterized by a latent time of 50 min, and the explosion time of the LP5 phage occurred at 60 min, burst size of 58 PFU/host cell (Figure 8).

Phages with high burst sizes and short latent periods are more effective to inactivate bacteria [36-53]. Phage LP8 showed the highest burst size and phage LP5 showed the lowest burst size. Latent periods of phages KP11, LP8 and LP5 was short (40, 30 and 50 min.) Tan et. al determined that the phages φH20 and KVP40 had similar burst sizes (70 and 80 PFU per cell, respectively) and identical latent periods (25 min) [43]. In another study, one-step growth experiments revealed that latent periods of 3 phages were 40, 90 and 120 min and each bacteria produced in order of 9, 15, 42 phages [36]. One-step growth experiments indicated that the five vibrio-phages were various in latent periods (10-70 min) and burst size (23-331 PFU per infected cell) [41].

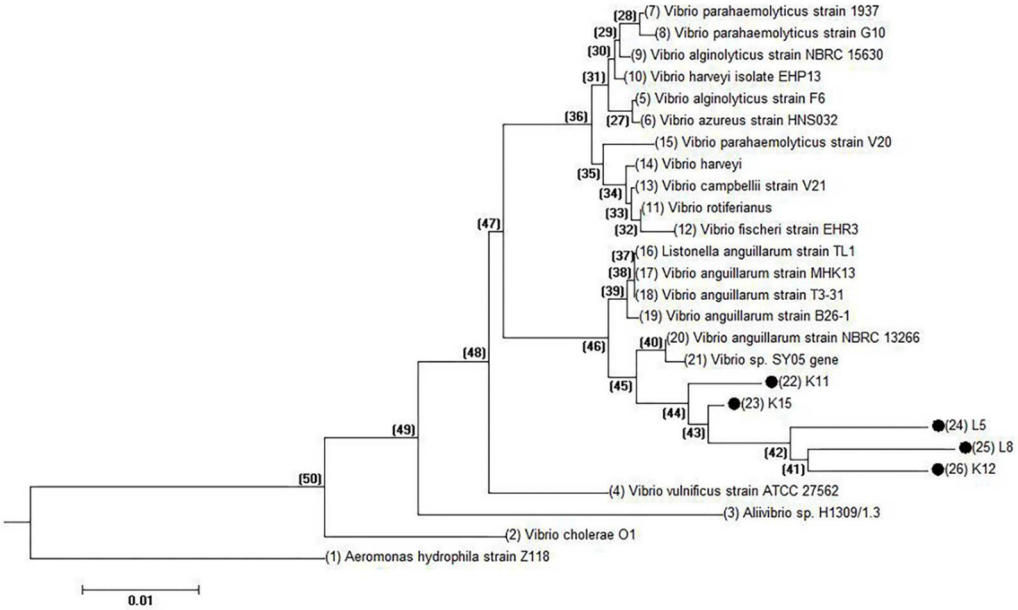

Figure 1: Phylogenetic tree with bootstrap values (n=500) showing the position of *Vibrio* sp. isolates relative to the related strains based on 16S rDNA sequences

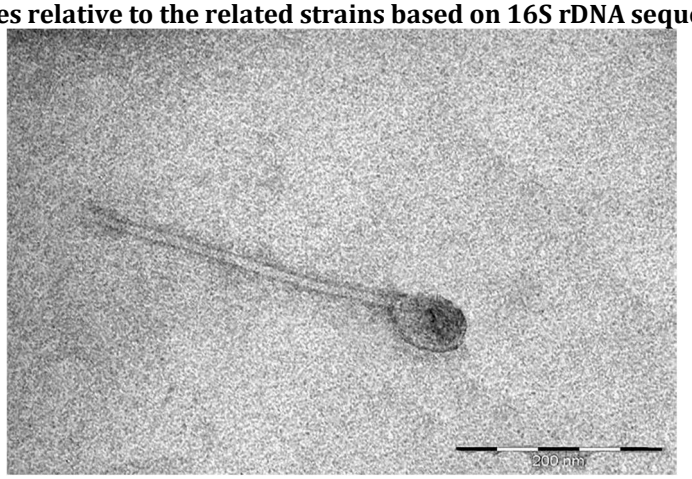

Figure 2: Transmission electron microscopy of Vibrio sp. bacteriophages KP11. The scale bar represents 200 nm. 200.000X magnification.

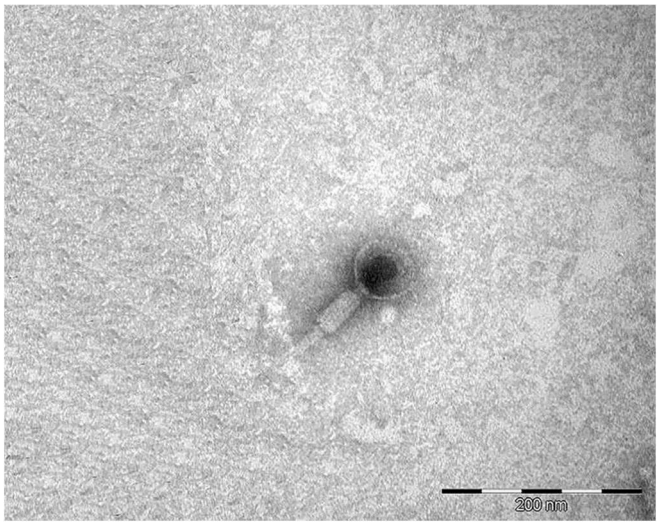

Figure 3: Transmission electron microscopy of Vibrio sp. bacteriophages LP5. The scale bar represents 200 nm. 200.000X magnification.

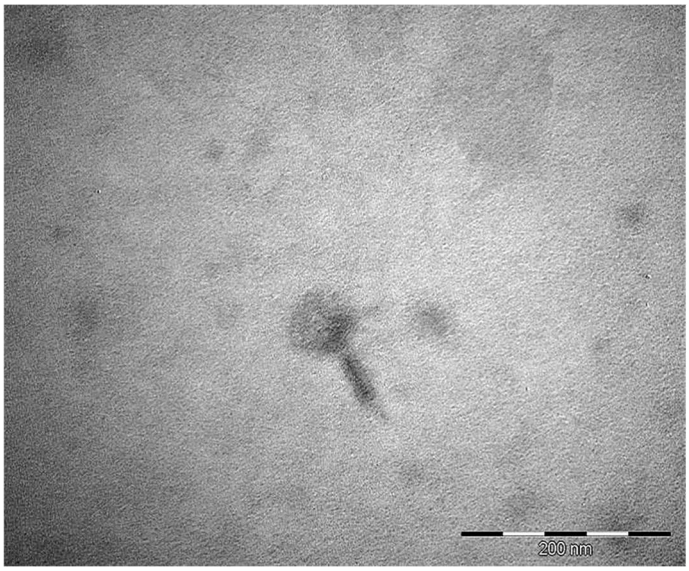

Figure 4: Transmission electron microscopy of Vibrio sp. bacteriophages LP8. The scale bar represents 200 nm. 200.000X magnification.

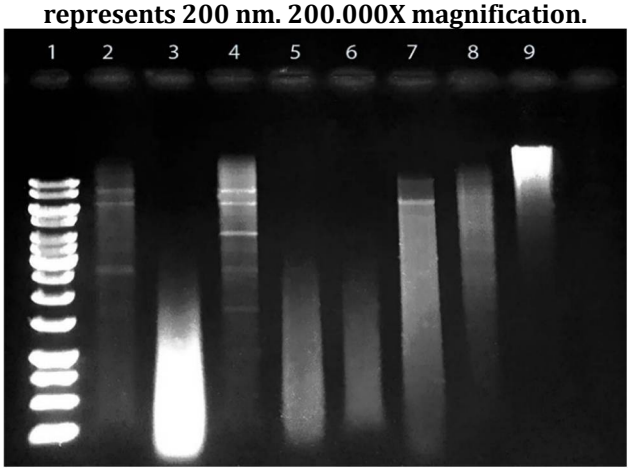

Figure 5: Restriction pattern of the bacteriophage genomes. Digestion of the genomes was performed with the enzyme HindIII. Line 1: kb DNA ladder, line 2: KP12 bacteriophage, line 4: LP8 bacteriophage, line 7: KP11 bacteriophage, line 8: LP5 bacteriophage. Lines 3, 5, 6 and 9 are uncut phage DNAs.

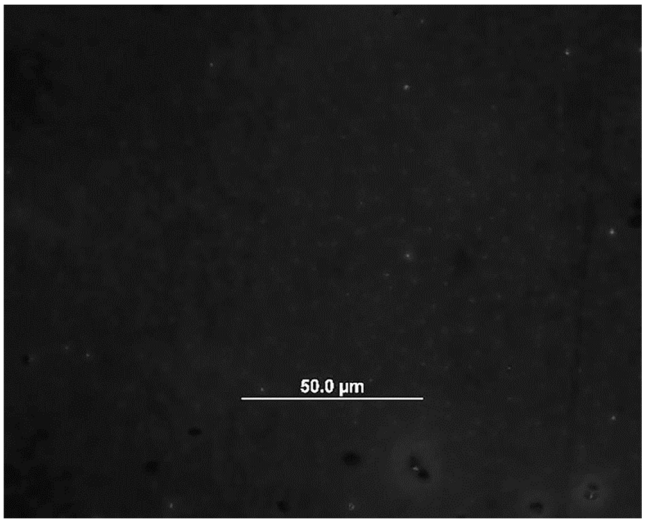

Figure 6: DAPI staining of bacteriophage suspension from sea water samples. Epifluoresence microscopy picture shows double-stranded DNA of phage particles appeared as tiny bright blue fluorescent dots on a dark blue background. Digital pictures were taken using 100X magnification objective.

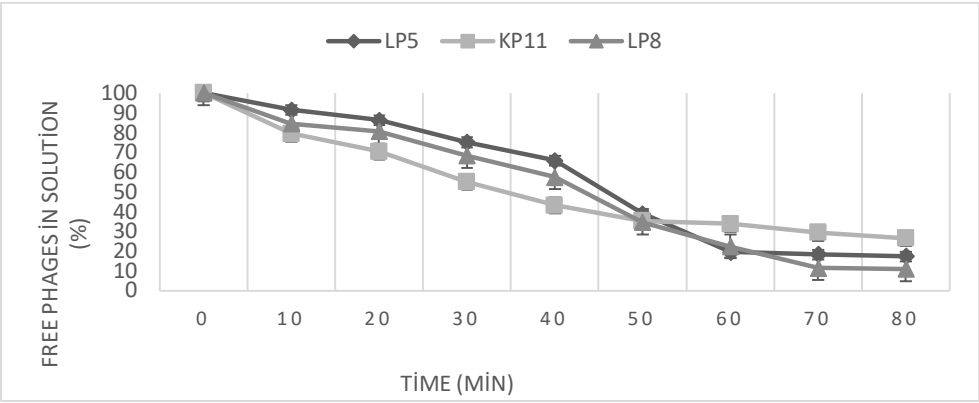

Figure 7: Adsorption assay of three phages KP11, LP8, LP5 on *Vibrio* sp. Strains in three independent experiment.

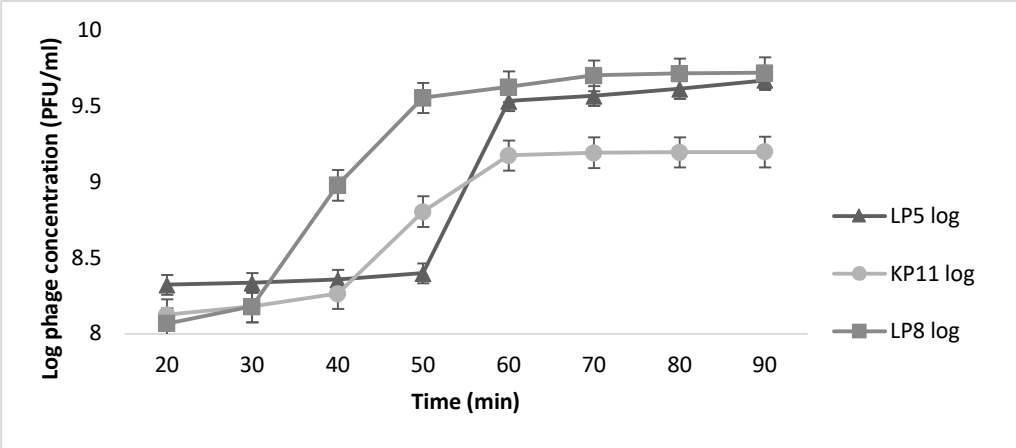

Figure 8: One-step growth curve of three phages in the three independent experiment; KP11, LP8, LP5 on *Vibrio* sp. strains at 25 °C.

Table 1. *Vibrio* strains isolated from sea bass

Strain	Source	Blast Result	Homology	NCBI Accession Number
K11	Sea bass, Country (Kidney)	*Vibrio anguillarum* strain 13266	96%	NR_113609.1
K12	Sea bass, Country (Kidney)	*Vibrio anguillarum* strain 4299 (Serotype O2b)	97%	CP011458.1
L8	Sea bass, Country (Liver)	*Vibrio anguillarum* strain MHK13	97%	KC884632.1
L5	Sea bass, Country (Liver)	*Vibrio anguillarum* strain SMP1 (Serotype O1)	97%	EF091702.1

Table 2. Some characteristics of bacteriophages recovered from *Vibrio* sp. strains

Host	Phage	Family	Genome size (kb)	Head diameter (nm)	Tail diameter (nm)	Tail length (nm)
Vibrio spp. L5	LP5	*Myoviridae*	44	55.5	18.9	44
Vibrio spp. L8	LP8	*Myoviridae*	73	66.1	17.9	73
Vibrio spp. K11	KP11	*Siphoviridae*	23	54.8	8.3	302.8
Vibrio spp. K12	KP12	-	55	-	-	-

Table 3. Host range and efficiency of plating of phages KP11, LP8, LP5 (+ plaques, - no plaques)

Host	KP11	LP8	LP5
	Spot test - Efficacy of plating (%)	**Spot test - Efficacy of plating (%)**	**Spot test - Efficacy of plating (%)**
Staphylococcus aureus (ATCC 25953)	–	–	–
Salmonella typhimurium (ATCC 14028)	–	–	–
Bacillus subtilis subsp. *spizizenii* (ATCC 6633)	–	–	–
Pseudomonas aeruginosa (ATCC 9027)	–	**+ 53,21%**	–
Escherichia coli (ATCC 25922)	–	–	–
Vibrio fischeri (ATCC 7744)	–	–	–
Vibrio sp. K11	**+ 100 %**	–	–
Vibrio sp. L5	–	**+ 82.27 %**	**+ 100 %**
Vibrio sp. L8	–	**+ 100 %**	–

CONCLUSIONS

In conclusion, fish infection by pathogenic bacteria is a progressive problem for the development of aquaculture worldwide. However, the increasing problem of antibiotic resistance in common pathogenic bacteria and concerns about spreading antibiotics in the environment, bring the need to find new methods to control fish pathogenic bacteria. The bacteriophages isolated in this study displayed lytic activity against strains of pathogenic *Vibrio* sp. Therefore, these vibriophages have potential for use as biocontrol agents to restain vibriosis in sea bass. However, further studies should be carried out to comprehensively demonstrate the effectiveness of these vibriophages as therapeutic agents in aquaculture.

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COMPETING INTERESTS

The authors have declared that no competing interest exists

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