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Phenotypic Characterization of *Vibrio* species: Application of Indigenous Phages for Biological Control of *Vibrio* in Aquaculture Live Feeds

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A B S T R A C T

Twenty *Vibrio* species were isolated from different sites along Alexandria sea shore. The phenotypic characterization, haemolytic activity and resistance pattern of the isolated *Vibrio* strains to different commercial antibiotics were investigated. All isolates showed varied results in the biochemical and physiological tests. Count estimation of the associated vibriophages were carried out using the isolated *Vibrio* species (V1-V20) as host strains. The highest counts (1840 PFU/ml) was recorded on V17. Four morphologically distinct phages namely P10, P16, P17 and P20 were selected and tested for their host specificity to the isolated *Vibrio* strains. P17 showed the highest host specificity (55%). The present study looks to sensitivity of P17 toward temperature, pH and ultraviolet radiation. Results concluded that P17 was sensitive to heat and the most destructive temperature was 5°C with 100% reduction in the phage titre. The highest lytic activity of p17 was at pH7, lower activity was observed at pH lower or higher than pH7. UV affected phage survival after 2 sec exposure, however low lytic activity was observed up to exposure to UV for120 sec. Phage host interaction showed that P17 had burst size (100 PFU per cell) and latent period (10 min). P17 was tested for its potentiality as biocontrol agent of *Vibrio* sp. in the live feed *Artemia salina*. P17 showed promising lytic activity against *Vibrio* sp. invading *A. salina* and recorded 92% reduction in *Vibrio* load after 18 h, which can be applicable as ecofriendly bio control agent of pathogens in the aquaculture.

Introduction

Bacterial infection is a significant threat to mankind. Many forms of human illness as a result of bacterial infection are common, with *Vibrio* species. *Vibrios* are one of the most common ubiquitous bacteria in the aquatic environment, they occur in commensal or symbiotic associations with eukaryotic organisms (Thompson *et al.,* 2004).

They naturally found in coastal, estuarine and marine environment world wide (Letchumanan *et al.,* 2014; Raghunath, 2014). *Vibrionaceae* have primarily been investigated due to their pathogenic potential to humans and aquatic animals and can be transmitted to humans via infected water or through fecal transmission. Several species of *Vibrio* have been implicated with disease in fishes (Elhadi *et al.,* 2004; Ran and Su, 2006; Su and Liu, 2007) and shrimp causing high mortality and serious loss in prawn hatcheries (Ruangpan and Kitao, 1991).

The presence of this bacterium in the marine environment raises the concern of human on food safety due to the latter potential in causing disease outbreaks depending on the environmental conditions (Ceccarelli *et al.,* 2013).

There are many different used antibiotics for treatment for *Vibrio* species infections (Han *et al.,* 2007; Al-Othrubi *et al.,* 2014). Our dependence on antibiotics to control bacterial infections in agriculture, aquaculture, veterinary medicine and humans resulted to indiscriminate use which in turn led to the emergence of multidrug resistant strains in the biosphere (Rao and Lalitha, 2015; (Letchumanan *et al.,* 2015; Shrestha *et al.,* 2015; Zavala Norzagaray *et al.,* 2015).

Development of novel non-antibiotic approach to fight against bacterial infections (Rice, 2008; Freire-Moran *et al.,* 2011) is needed. Recently, interest in the application of bacteriophage to control bacterial infections in various fields including agriculture, veterinary, food safety and human infections has been renewed (Meaden and Koskella, 2013; Payet and Suttle, 2014; Wittebole *et al.,* 2014).

Early discovery of bacteriophages as antibacterial agents were reported in 1896 after observing antibacterial properties of this viral like agent against *Vibrio cholerae* in Ganges River, India (Adhya and Merril, 2006).

Therefore, the present study aims to isolate, characterize different *Vibrio* species and their associated phages and provides an insight into *Vibrio*phages and their interaction with their *Vibrio* host species. Moreover, bacteriophage application in controlling *Vibrio* species in *Artemia salina* before their administration to fish larvae will be investigated.

Materials and Methods

Sampling

Water samples were collected from different sites of Alexandria sea shore, Egypt, in a sterile screw capped bottles, transferred to laboratory in ice box according to APHA (1998) and stored at 4^oC till analysis.

Culture media

All culture media which were used for isolation were of pure grade and purchased from Difco, Detroit, USA, and prepared according to the manufacturer's instructions.

Isolation of *Vibrio* species

Water samples were diluted up to 10^{-5} with sterile sea water., spread on TCBS (Thiosulfate Citrate Bile Salts Sucrose) agar plates, and incubated at 30°C for 48 h. Representative colonies were picked and transferred onto marine agar (MA; BD Difco) plates for further purification and taxonomic studies (Abou-Elela *et al.,* 2009a)

Phenotypic characterization

Twenty *Vibrio* isolates were picked from TCBS agar plates according to their colony morphology, size, and pigmentation variability and examined for some morphological characters on nutrient agar after 24 h. Physiological and biochemical tests such as temperature in the range of $10–40^oC$ were studied. Tolerance to NaCl was determined by the addition of NaCl with 6, 8 and 10 %. A number of conventional biochemical tests were carried out on all isolates, including sugar fermentation, urease, catalase, gelatinase and indole production, degradation of agar, starch, casein. Antimicrobial test was carried out according to El-Masry *et al.,* (2002).

Haemolytic activity

Haemolytic activity was performed using human blood agar plates 5 % (v/v). Positive result was indicated as clear zone of haemolysis around the colony (Brender and Janda, 1987).

Antibiotic susceptibility test

Resistance of the isolated *Vibrio* species were tested against different antibiotics (imipenem, 10 µg; ampicillin, 10 µg; norfloxacin 10 µg; cephalexin, 30 µg; erythromycin, 15 µg; flucloxacillin, 5 µg; ciprocin 5 µg and chloramphenicol, 5 µg, by disk diffusion method (Bauer *et al.,*1966). Selected antibiotic discs were placed on Mueller Hinton Agar (HiMedia, India) (with 2% NaCl) plates seeded with bacteria. These plates were then incubated at 37°C for 24 hours. The organisms were observed for antibiotic sensitivity based on diameters of zones of inhibition on the Petri dishes.

Estimation of phage counts infecting *Vibrio* species

Double agar layer technique (DAL) described by Adams (1959) was used for phage detection and enumeration. One ml of the water sample or appropriate dilution of the sample and 0.2 ml of exponentially growing host culture were added to 3 ml of liquefied soft agar. The mixture was poured onto Petri dishes containing nutrient agar medium, allowed to solidify and incubated at 30°C. The plaques were counted following 16-18 hours incubation.

Isolation, propagation and purification of bacteriophages

Bacteriophages specific to the isolated *Vibrio* strains were isolated from seawater samples using a double-agar-layer method (Adams, 1959). The plates were incubated overnight at 25°C for plaque formation. If plaques were detected, a single plaque was picked from the plate using a sterile Pasteur pipette tip and eluted into 1 mL of exponential phase culture of the respective *Vibrio* strain. The mixture was plated on semi-solid agar medium again for plaque formation. The procedure was repeated three times for phage purification. Purified phages were stored in SM buffer (50 mM Tris-HCl, pH 7.5, 99 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin) at 4°C.

Determination of phage titre

Titers of phage suspensions were determined by serial dilution in SM buffer followed by a drop count method. One milliliter of exponential phase bacterial culture was mixed with 5 mL of warm soft nutrient agar and then poured over presolidified nutrient agar plates to prepare semi-solid agar plates. Five µl drops of each phage suspension dilution were inoculated on the surface of semisolid agar plates (Yu *et al.,* 2013).

The inoculated plates were incubated overnight at 25°C for plaque formation. All treatments were performed in triplicates. The number of plaques in each drop was recorded, and tires of phage suspensions were defined as plaque-forming units (PFU/ml).

Determination of lysis spectrum of the isolated bacteriophages

All the isolated *Vibrio* species were used for determination of lysis spectrum of the isolated bacteriophage. Briefly, 5 µl of each isolated phage were spotted on each agar plate with different *Vibrio* strains. The plates were incubated at 25 °C overnight and examined for the appearance of plagues (Taj *et al.,* 2014).

Electron microscopy examination

A suitable volume of concentrated bacteriophage sample was deposited on a Formvar-carbon-coated copper grid. The

samples were negatively stained with 2% sodium phosphotungstate (pH 7.6) and then examined with JEOL 100 CX transmission electron microscope (TEM) operating at 80 kV at Faculty of Science, Alexandria University (Stenholm *et al.,* 2008).

Heat sensitivity

The thermal stability of phages was examined by pre incubating phage suspensions at different temperatures (5, 30, 50, 70 and 100°C, respectively) at pH 7 for 2 h. The phage suspensions were immediately cooled in ice water, and the surviving phages were estimated by the double-agar layer method (Basdew and Laing, 2014).

pH sensitivity

The pH stability of phage was examined by pre-incubating the phage suspensions at different pH levels (4, 7, 9, 11, respectively) at 30°C for 2h. The surviving phages were immediately estimated by the double-agar layer method (Basdew and Laing, 2014).

Ultraviolet irradiation sensitivity

Sensitivity of phage isolate to ultra violet irradiation was done at a distance of 30 cm from a 15 Watt sterile lamp for different time intervals. Survival of phage isolate was determined at each time interval using the double-agar layer (Hassan, 2002).

Statistical analysis

Data analysis was performed with the software package Microsoft Excel, Version 2003. Statistically significant difference was determined using paired Student's t-test and $P<0.001$ was used as a limit to indicate statistical significance.

One-step growth curve (phage-host interaction)

One-step growth curve experiments were adapted from Adams (1959) with modifications to determine the latent time and phage burst size. Phage suspension was mixed with 1 mL of exponential phase culture of *V. parahaemolyticus* and incubated at 25°C for 10 min for phage adsorption. The mixture was centrifuged at 10000 g for 10 min to remove free phage particles. The pellet was suspended in 60 mL of broth, and the culture was continuously incubated at 25°C. Samples were taken at 10 min intervals upto 2 h, and phage titer was determined (Yu *et al.,* 2013).

In vivo administration of phage in *A.salina* culture

The brine shrimp, Artemia is a zooplanktonic organism widely used as live feed. It can be hatched within 24 hours from dormant cysts (batch culture) which can be easily distributed and stored for prolonged periods of time. Plastic containers were used, each containing 1L of *A. salina* cultures supplied with intense aeration. *A. salina* was dosed with phage P17 (10^{11} PFU/ml) while the control kept without phage inoculation. The total presumptive *Vibrio* count in each container was assessed at different time intervals, following serial dilutions of 1 ml samples and plating in TCBS. The experiment was done in triplicates (Kalatzis *et al.,* 2016).

Results and Discussion

Isolation and phenotypic characterization of *Vibrio* spp.

Vibrio spp. occurs naturally in aquatic environments and are one of the most commonly-occurring bacteria during shrimp farming (Vandenberghe, *et al*, 2003). The number of reported *Vibrio* species increased rapidly in the last decade (Thompson, *et al.,* 2004). *Vibrio* species have been associated with skin infections and severe gastrointestinal disorders (Andrews, 2004). In the present study, among the isolated *Vibrio*

spp., twenty isolates were selected according to difference in size, colony morphology and pigmentation. They were subjected to morphological, physiological and biochemical characterization (Table 1). According to the tested characters, all the isolated *Vibrio* species were grouped into 3 phena.

Phenon A

This phenon contained 4 strains. They grew well at temperature range (30-40 ºC) but without growth at 10 ºC and 20°C. All strains grew at 6, 8% Na Cl while only two grew at 10%. They were able to utilize glucose, fructose and sucrose as carbon source, moreover they had capability to produce catalase, urease and gelatinase, but no indole production or nitrate reduction. They were able to degrade starch only. Antibacterial activity was observed against all tested bacterial pathogens except for *P. aeruginosa* and *V. anguillarum*.

Phenon B

This phenon harbored 4 strains, they are characterized by well growth at temperature range (30-40 ºC). All strains grew at 6% NaCl, 75% of the tested strains were able to grow at 8 and 10% NaCl. No indole production or nitrate reduction. They were fermenters of glucose and sucrose but no fermentation of fructose. They exhibited the ability to degrade starch and casein. All isolates showed antibacterial potentiality against the tested pathogens except for *V. anguillarum*.

Phenon C

This phenon was the major one and contained twelve strains. All strains grew at temperature (30-40 ºC). 75% of the tested strains grew at 6 and 8% NaCl, while only 50% were able to grew at 10%. Contrarely to phenon A and B, all the tested *Vibrio* species in phenon C were positive for indole production and nitrate reduction. They are fermenters of glucose and fructose, only 92% are sucrose fermenters. Varied percentages of antibacterial activity were detected against the tested pathogenic bacteria with no antibacterial activity against *P. florescence, P. aeruginosa, V. anguillarum* and *E. coli.*

Taxonomic study of *Vibrio* spp. was previously reported in different studies (Noriega-Orozco *et al.,* 2007, Abou-Elela, *et al.,* 2009b; Ganesh *et al.,* 2012).

Haemolytic activity

Haemolytic activity of the isolated *Vibrio* species was tested on human blood agar. Results in table 2 revealed that haemolysis of human blood were detected in 12 isolates representing about 60% of the isolated strains.

Antibiotic resistance of the haemolytic *Vibrio* spp.

Variation in the *in vitro* susceptibility of *Vibrio* to antibiotics has been observed, with emerging resistance to nalidixic acid, co-trimoxazole, furazolidone and streptomycin. The emergence of multidrug-resistant strains has been a matter of concern (Mahbub *et al.,* 2011). The presence of multiple antibiotic resistances in isolates that are not recognized as pathogens is also extremely important, as this indicates that commensal or environmental bacteria can serve as reservoirs for resistance genes.

In the present study, The haemolytic *Vibrio* spp. were screened for the sensitivity to some commercial antibiotics including imipenem,10 μg; ampicillin, 10 μg; norfloxacin,10 μg; cephalexin, 30 μg; erythromycin,15 μg; flucloxacillin, 5 μg; ciprocin, 5 μg, and chloramphenicol, 5μg. As shown in table 3 out of the tested 12 strains, eight strains were resistant to

chloramphenicol 5µg, ampicillin10 µg and erythromycin, 15 µg which represented the highest resistance percentage (67%). On the other side, flucloxacillin, 5 µg was the most effective against about 58% of the tested strains. Different degree of resistance was observed for the other tested antibiotics which agree with that reported by Abou-Elela *et al.,* 2009a. In comparative study, Mahbub *et al* (2011) studied the antibiotic resistance pattern of fifteen *Vibrio* spp. isolated from shrimp rearing ponds in Bangladesh and it was shown that the highest resistance was to ampicillin (100%), followed by amoxicillin (78%), nalidixic acid (40%), vancomycin (13.33%), neomycin (6.66%) and chloramphenicol (6.66%). Similar observation was reported by Ransangan *et al.,* (2013) where approximately 78% of the environmental *Vibrio* isolates were found to be resistant to ampicillin. Similarly, Manjusha *et al.,* (2005) reported that environmental *Vibrio* isolates were the most resistant to ampicillin and all the isolates were observed as sensitive to gentamycin, erythromycin, ciprofloxacin and doxycycline. Ransangan *et al.,* (2013) reported the sensitivity of all tested *Vibrio* spp. to chloramphenicol.

Diversity of *Vibrio*phages in the selected water samples

Successful application of phage therapy in the treatment of *Vibrio*sis requires detailed knowledge of the diversity and distribution of the associated phages and susceptibility properties of *Vibrio* species as well as a collection of well-characterized phages that covers host diversity (Taj *et al.,* 2014).

In the present study, diversity of the indigenous phages infecting *Vibrio* spp. was studied in the collected samples. All the isolated *Vibrio* species (V1-V20) were used as host culture for detection and estimation of phage counts in the selected samples. As shown in figure 1, the counts of *Vibrio* phages ranged from 4x10 to 184x10 PFU/ml. The highest count (184x10 PFU/ml) were detected on V17 and recorded as the most sensitive strain to *Vibrio*phages followed by V16 which recorded 56x10 PFU/ml, while the lowest count was detected on V4 with only 40 PFU/ml. On the other side, no estimates of bacteriophages were detected on V1, V5, V6, V7, V8, V9, V11, V12 and V15.

Determination of the lysis spectrum of the isolated bacteriophages

Among all isolated phages, four morphologically distinct *Vibrio*phages namely P10, P16, P17 and P20 were selected and tested for their lytic capability or specificity toward all the isolated *Vibrio* spp using spot assay method. As shown in table 4, P10 showed narrow range of host specificity and exhibited lytic activity against only V2, V4, V15, V16 and V10, which represented 25% of the tested *Vibrio* species. Also V16 and V20 showed narrow range of host specificity with 5 and 30% infection capability, respectively. Narrow range of host specificity can be explained by the fact that most of the marine phages are specific and lyse only the original host bacterium as was described by Hassan, 2011. On the other side, P17 showed broader lytic capability and host range where it was able to infect V2,V4, V8,V9, V10, V12, V14, V15, V16, V17 and V20 which represented 55% of the tested *Vibrio* species and was not limited to the host strain, The most sensitive bacterial isolate V17 was selected to complete the study and identified as *Vibrio parahaemolyticus* by using API 20 Kit. *Vibrio parahaemolyticus* is a halophilic gram-negative bacterium that is widely distributed in coastal waters worldwide and is associated with wound infections, gastroenteritis, and septicemia (Daniels *et al.,* 2000). Occurrence of *V. parahaemolyticus* infections are reported to occur due to direct contact with estuarine waters or the consumption of undercooked raw shellfish (Lin and Lin,

2012). *V. parahaemolyticus* pandemic strains have displayed multiple antibiotic resistances, increasing concerns about possible treatment failure. Yu *et al.,* (2013) studied the host range of five isolated *Vibrio* phages (P3K; P4A; P7A; P8D; P9C) against different *Vibrio* strains isolated from sea water samples and reported that most of the *Vibrio* host strains lost their sensitivity against these five phages. So, the highest resistance with 97% was against phage P9C and the lowest resistance was found with 41% against phage P3K. This lytic spectrum could be explained by the conservative nature of the structure of phage receptors on the outer membrane of many of Gram negative bacterial species (Rakhuba *et al.,* 2010). Regarding the host specificity of VP17, it was selected for more detailed studies of phage lytic potential and phage-host interactions.

Characterization of P17

Electron microscopic examination

The electron micrograph of P17 is illustrated in Figure 2. Based on particle morphology, P17 belonging to order Caudovirales, and family Podoviridae which is characterized by icosahedral capsid and short tail. To date, most of the marine viruses reported are bacteriophages that belong to order *Caudovirales* (Rao and Lalitha, 2015, Letchumanan *et al.,* 2016). Podoviruses were previously isolated from marine environment (Jiang *et al.,* 1998; Sun *et al.,* 2014; Zhan *et al.,* 2016). Figure 3 represents the shape of plaques produced by phage P17 on the host bacterium (*V. parahaemolyticus*). As shown, the plaques were of about 3mm diameter with clear center

Heat sensitivity

Temperature is one of the most important environmental factors that strongly affect many aspects of the biological systems. Influence of changes in temperature regimes on the biological system is very vivid and affects the species distributions, evolution of phenotypic traits (Vale *et al.,* 2008). P17 was exposed to different temperature for a period of 2 h min before addition to the host bacterium. The counts of P17 were estimated each time by the use of DAL. Significant differences were noted between phage activity at each temperature (P<0.001), p17 was sensitive to temperature and its sensitivity increased by increasing temperature, however it still active even after boiling, the optimum temperature for bacteriolytic activity was $3x10^8$ PFU/ml at 30 ^oC (Figure 4). Overall results show that propagation of these phages is negatively affected by increased exposure to high temperatures and the most damaging temperature was 5 ^oC with 100% reduction in phage titers followed by reduction of phage counts to $97x10^5$ PFU/ml up on exposing to 50^oC. This result is encouraged by study of Jun *et al.,* (2014) who observed that pVp-1 phage specific to *V. parahaemolyticus* was relatively heat stable over a temperature range of 20°C–37°C, although decrease in phage activity was detected at 50°C and complete loss of activity was at 65°C. Basdew and Laing (2014) reported that increase in temperature decreases virus survival and activity and exposure to 70°C was the most damaging with a 92 to 96% reduction in phage titers. In the same way, findings by Pope *et al.,* (2004) indicated an increase in bacteriophage yield till 30°C and 39°C which corroborates the present study. Lal *et al.,* (2016) isolated VpKK5 as specific phage to *V. parahaemolyticus* and found that it was stable at 40 °C and declined at 50 °C following heating for 60 min, moreover the activity was disappeared entirely when heated at more than 60 °C for 1 h.

pH sensitivity

pH is an important factor which make changes in the protein of the tail region of the

phage particle and could affect the adsorption of the phage to the bacterial wall, thus inactivating the phage particle (Seeley and Primrose, 1982). ANOVA showed highly significant differences ($p<0.001$) between phage titres treated with different pH ranges. The present study (Figure 5) concluded that pH7 was the most suitable for bacteriolytic activity of P17 which recorded $3x10^8$ PFU/ml followed by pH 9 with $1x10^7$ PFU/ml, however low stability of P17 is still present until pH 11 which recorded $2x10^4$ PFU/ml. On the other hand, low pH 4 caused reduction in the phage counts to $2x10^5$ PFU/ml. pH finding of the study was confirmed by Langlet *et al.,* (2007) and Ibrahim *et al.,* (2017) who indicated that virus exhibited stability at wide range of pH regimes. The same finding was reported by Taj *et al.,* 2014.

In accordance with the present study, pH 6 to 7 was reported for optimal phage replication, followed by a sharp decline at higher pH (Basdew and Laing, 2014). This was also noted in a study by Da Silva and Janes (2005), where phages specific to *Vibrio* spp. (infective on oysters) were screened at various pH ranges (6-7) and it was noted that *Vibrio* phages were most stable at that pH range which best mimicked the pH of the oyster system. Lal *et al.,* (2016) reported that the activity of the *Vibrio* phage VpKK5 was measurable after incubation at pH 4–9, but completely disappeared at pH 2 and pH 3.

UV sensitivity

Ultraviolet radiation as a mean of reducing microorganisms is gaining more importance both in the treatment of certain drinking water and in advanced treatment of wastewater (Zukovs *et al.,* 1986). As observed in Figure 6, P17 was affected by the exposed UV and the phage titre was reduced to $4x10^5$ after 2 sec, and the counts reduced with increasing the time of exposure to reach $3x10^3$after 60 min., however the phage is still having bacteriolytic activity with $2x10^5$ PFU/ml until exposure to 120 sec. ANOVA showed highly significant differences ($p<0.001$) between phage titres treated with UV for different time intervals. Hassan, (2002) reported that some isolated bacteriophages were stable upon exposure for 30 sec. but complete activation was observed at 60 and 120 sec. Studies on λ phage (Wiegle, 1953) reported the inactivation of a large proportion of the phages upon exposure for 60 sec and a high frequency of mutation in the surviving phage particles. Time required for the 37% survival of the *V. cholera* S20 phage was 7 sec. Around 80% phage particles were inactivated within 10 sec of exposure to UV and only 10% survival was at 16 sec as was reported by Dutta and Ghosh, 2007. A complete inactivation of *V. parahaemolyticus* phage Vp-1 was approximately at 45 mins upon exposure to UV light, as was observed by Jun *et al.,* 2014.

Phage-host interaction

Phage host interactions are essential requirements for phage applications (Tan *et al.,* 2014). Life cycle characteristic was determined for phage P17 from one-step growth curve during incubation with *V. parahaemolyticus*. As shown in figure 7, P17 exhibited the burst size (100 PFU per cell) and latent period (10 min). Phages with high burst size are the most appropriate candidates for phage therapy. Thus, P17 can be considered as good candidates for phage therapy applications since it has relatively high burst size compared to studies of Phumkhachorn and Rattanachaikunsopon, (2010) who reported that the latent period of the *Vibrio* phage PW2 was 30 min and it has a burst size of 78 PFU per infected cell. Lin *et al.,* (2012) stated that he burst size of phage A318 infecting *V. alginolyticus* was 72 PFU per infected cell. Short latent period of approximately 15 min with a burst size of 47 PFU/cell was for *V. parahaemolyticus* phage Vp-1 as was stated by Jun *et al.,* 2014. On the

other side, Lal *et al.,* (2016) reported that the latent period of the *Vibrio* phage VpKK5 was 36 min with 180 PFU per infected cell as burst size. Another study recorded long latent periods (150 and 180 min) for two bacteriophages isolated from the North Sea (Chan *et al.,* 1966). Børsheim (1993) suggested that there is great variation in burst size; the average marine phage burst size range from 5 to 610 and the latent periods may increase to 170 and 120 min as was stated by Zachary, 1978. It can be concluded that the burst size and latent period of P17 isolate fell within the documented range for marine phages.

In vivo efficacy of P17 to control *Vibrio* load in *A. salina* culture

Increase of the pathogen load could increase the possibilities of disease outbreak in aquaculture. Effective prevention strategies will be essential in reducing disease burden due to bacterial infections (Yen *et al.,* 2017). Reducing and controlling of *Vibrios* in fish and invertebrates hatcheries is critical for the survival and quality of the produced larvae. Live feed organisms like *Artemia* are able to bio-accumulation of bacteria from the water column acting as a vehicle for pathogen transfer into the hatchery facilities. Currently, the need of an innovative and environmentally friendly alternative to antibiotics has become more than necessary. Presence of bacteriophages in environments with pathogenic bacteria is an opportunity for development of a successful innovative and environmentally friendly solution for the prevention of multi-drug resistant bacteria spreading in aquaculture (Nakai and Park, 2002).

Fig.1 Counts of vibriophages on the isolated *Vibrio* species (host bacteria)

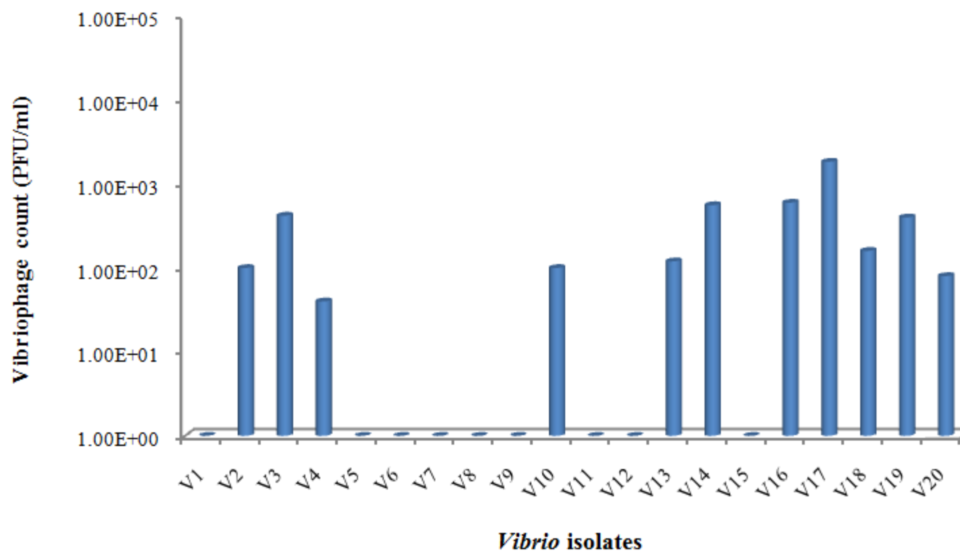


Table.1 Phenotypic characterization of the isolated *Vibrio* spp

Characters	Phenon A 4 strains	Phenon B 4 strains	Phenon C 12 strains
Morphological characters			
1. Colour			
Yellow	25	0	25
Green	75	100	75
2. Cell shape			
curved-rod shape (Comma shape)	100	100	100
3- Diffusible pigments	0	0	0
Physiological characters			
1- Growth at different Na Cl concentrations (%)			
6	100	100	75
8	100	75	75
10	50	75	50
2- Growth at different temperatures (^{o}C)			
10	0	0	0
20	0	0	0
30	100	100	100
35	100	100	100
40	100	100	100
Physiological characters			
Production of			
Catalase	25	25	67
Urease	75	75	92
Indole	0	0	100
Gelatinase	25	75	25
Nitrate reduction	0	0	50
Utilization of			
Glucose	100	75	100
Fructose	75	0	100
Sucrose	50	25	92
Degradation of			
Agar	0	0	0
Starch	100	100	58
Casein	0	100	25
Antibiosis against			
Pseudomonas. aeruginosa	0	100	0
Staphylococcus.aureus	75	50	50
Aeromonas. hydrophila	50	50	58
Vibrio.anguillarum	0	0	0
Pseudomonas fluorescence	50	50	0
Escherichia.coli	25	50	0

Table.2 Haemolytic activity of the isolated *Vibrio* spp

Vibrio isolates	Haemolysis
V1	+
V2	+
V3	+
V4	+
V5	+
V6	+
V7	+
V8	+
V9	+
V10	-
V11	-
V12	-
V13	-
V14	-
V15	-
V16	+
V17	+
V18	-
V19	+
V20	-

Table.3 Resistance of the haemolytic *Vibrio* isolates to different commercial antibiotics

Tested antibiotics	**Haemolytic *Vibrio* isolates**											
	V1	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**	**V9**	**V16**	**V17**	**V19**
Chloramphenicol,5 µg	+	-	-	+	-	+	+	+	+	+	+	-
Flucloxacillin,5 µg	-	-	-	+	-	-	+	+	+	+	-	-
Ciprocin, 5 µg	+	-	-	+	+	+	+	-	+	-	-	-
Ampicillin, 10 µg	+	+	-	+	+	+	+	-	+	-	-	+
Cephalexin, 30 µg	+	-	-	+	+	+	+	-	+	-	-	-
Erythromycin, 15 µg	+	-	-	+	+	+	+	+	+	-	-	+
Norfloxacin,10 µg	+	-	-	+	+	-	-	+	+	+	+	-
Imipenem,10 µg	-	+	+	+	+	-	+	+	+	-	-	-

+: Resistance; -: Sensitivity

Table.4 Host range of *Vibrio* phages P10, P16, P17 and P20 against the isolated *Vibrio* species

***Vibrio* host**	**Phage isolates**			
	P10	P16	P17	P20
V1	-	-	-	-
V 2	+	-	+	-
V 3	-	-	-	-
V 4	+	-	+	-
V 5	-	-	-	-
V 6	-	-	-	-
V 7	-	-	-	-
V 8	-	-	+	-
V 9	-	-	+	+
V 10	+	-	+	-
V 11	-	-	-	+
V 12	-	-	+	-
V 13	-	-	-	-
V 14	-	-	+	-
V 15	+	-	+	-
V 16	+	+	+	+
V 17	-	-	+	+
V 18	-	-	-	+
V 19	-	-	-	-
V 20	-	-	+	+

Fig.2 Electron micrograph of P17

Fig.3 Plaques produced by phage P17 on *V. parahaemolyticus* host bacterium

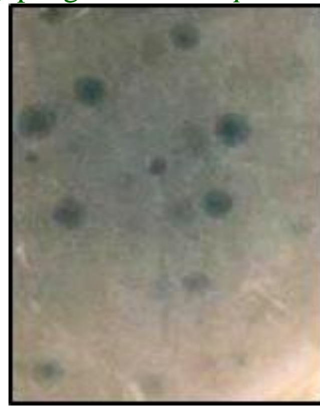


Fig.4 Effect of temperature on survival of phage P17. Different letters (a, b,c) indicate significant difference ($p < 0.001$)

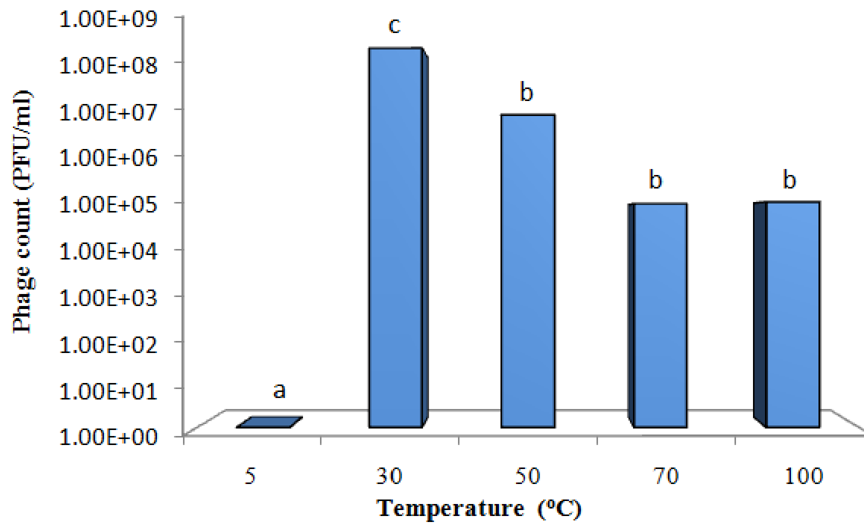


Fig.5 Effect of pH on survival of phage P17. Different letters (a, b, c) indicate significant difference ($p < 0.001$)

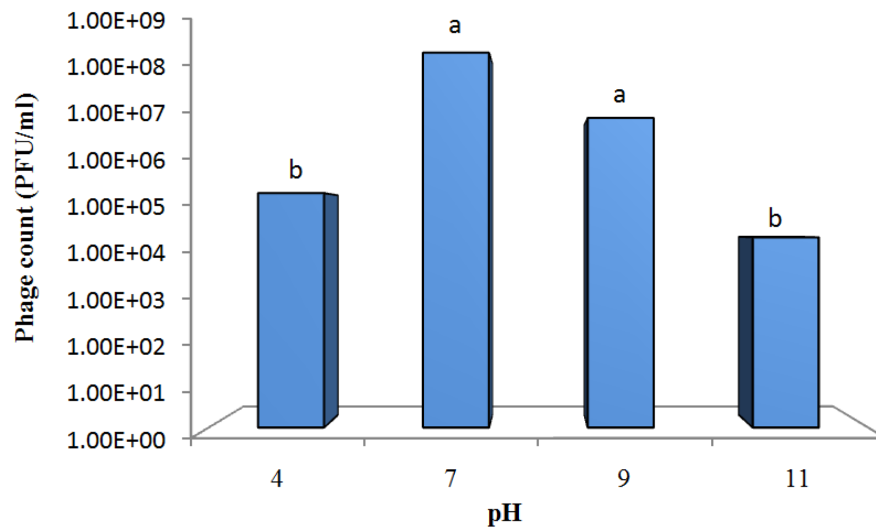


Fig.6 Effect of UV on survival of phage P17. Different letters (a, b, c) indicate significant difference ($p < 0.001$)

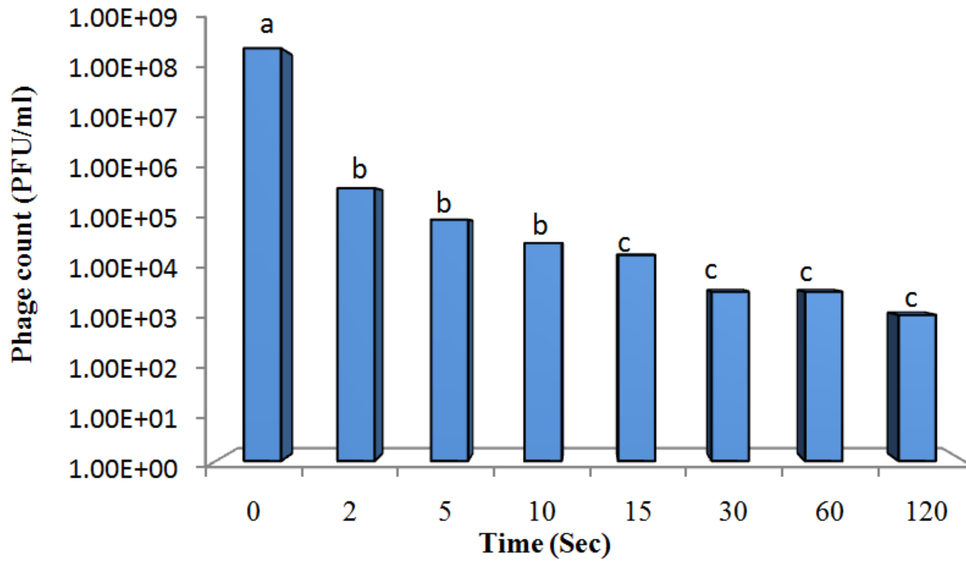


Fig.7 One-step growth curve of phage P17 infecting *V. parahaemolyticus.*

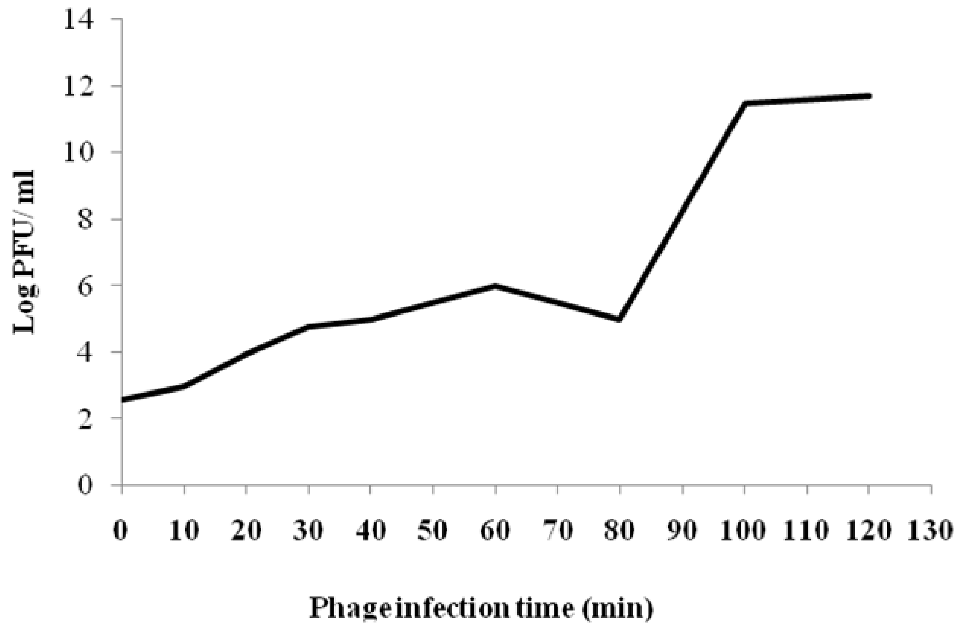
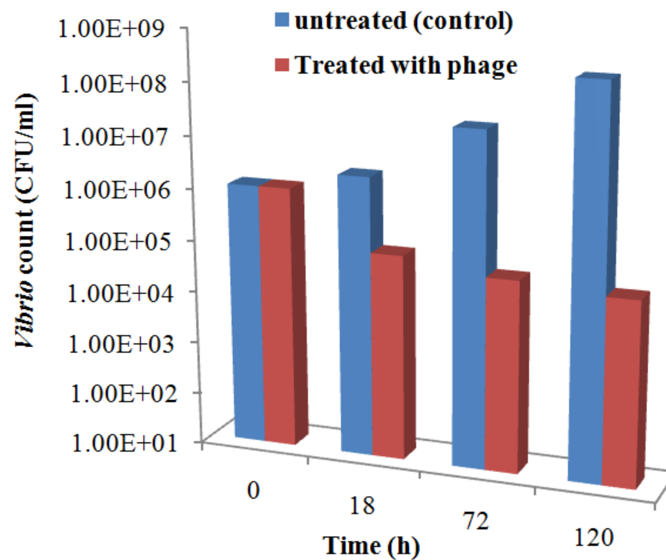


Fig.8 In vivo efficiency of phage P17 in elimination of *Vibrio* spp. in *A. salina* culture



The current experiment aimed at evaluating the *in vivo* efficiency of P17 in controlling *Vibrio* load. To achieve this goal, culture of *A. salina* was dosed with P17 (10^{11} PFU/ml) and counts of *Vibrio* spp. was monitored at different time intervals for 120h (Figure 8). The present study confirmed the possibility of using P17 as biocontrol agent of *Vibrio* spp. in *A. salina* culture, where the initial presumptive *Vibrio* load ($11x10^5$ CFU/ml) in phage treated *Artemia* was decreased by 92% after 18 h to reach ($8.8x10^4$ CFU/ml) and 95% after 72 h to reach ($5.5x10^4$ CFU/ml) and the antibacterial effect of P17 was prolonged even after 120 h with 97% inhibition of *Vibrio* load to be eliminated to ($3.3x10^4$ CFU/ml). The brine shrimp, *Artemia* is a zooplanktonic organism widely used as live feed. It can be hatched within 24 hours from dormant cysts (batch culture). The efficiency of P17 to eliminate *Vibrio* load after 18 h is an advantageous as treatment in the live feeds prior to their introduction in the hatchery system and could effectively reduce the *Vibrio* load in the larval rearing tanks, which in turn can reduce *Vibrio* load in fish hatcheries. This finding is supported by a study of Kalatzis *et al.,* (2016) who confirmed that invivo administration of the phage cocktail, φSt2 and φGrn1 on live prey *A.salina* cultures, led to a 93% decrease of presumptive *Vibrio* population after 4 h of treatment. Pai (2006) isolated five *Vibrio* phages from a sediment sample collected from the Vembanad estuary, Kochi. Kerala and evaluated their efficiency to prevent the growth of *V. harveyi* in plain seawater and concluded that phages were able to arrest the growth of *V. harveyi* for about l2 h after which phage resistant forms emerge and begin to dominate. Numerous studies using bacteriophages as treatment agents, verify their applications in many aquaculture systems have been successful against many pathogenic bacteria such as *V. parahaemolyticus, V. harveyi,* and *V.anguillarum* (Vinod *et al.,* 2006; Karunasagar *et al.,* 2007; Higuera *et al.,* 2013; Lomelí-Ortega *et al.,* 2014).

In conclusion, the *Vibrio*phage P17 could be promising antibacterial candidate against pathogenic bacteria and can be used as effective alternative to the commercial antibiotics. Future studies are needed to characterize the genome of P17 and to establish its potential application for *Vibrio* treatment on large scale.

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