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Research Article

Characterization of *Vibrio alginolyticus* bacteriophage recovered from shrimp ponds in south west of Iran

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Abstract

Farming of the pacific whiteleg shrimp (*Litopenaeus vannamei*), consists the greatest percent of aquaculture production of crustaceans all over the world. Vibriosis caused by *Vibrio* species such as *Vibrio alginolyticus*, is a serious limiting agent for aquaculture. Phage therapy is an eco-friendly alternative to antibiotics as a prophylactic measure for the growth of pathogenic bacteria. The aim of this study was isolation and characterization of *Vibriophages* present in *L. vannamei* breeding and culture facilities and estuarine resources in south west of Iran, Choebdeh shrimp farming site of Abadan city, Khuzestan Province, based on morphological structure, biological conditions and their potential applications in lysis of *Vibrio* bacteria. The bacterial and phage samples were analyzed using different physicochemical parameters, 16S rRNA gene sequencing and TEM. The results showed the *V. alginolyticus* isolates and identified the *Podoviridae* family of phages. These bacterial isolates were resistant to ampicillin and penicillin. The efficacy of the *Podoviridae* phage against *V. alginolyticus* bacterium indicated that this phage has a short period of 30 minutes latent phase, possessed a desirable stability and growth in keeping for 1 hour at 30-55^{o}C, the salinity of 0.5-10‰ and pH of 5-8. Based on evaluation of phage therapy, mean absorbance values (OD_{600}), in control and treatment groups, at 12 and 24 hours after phage inoculation, were significantly different (p<0.05). According to the results, phage therapy with single-phage suspensions did not prevent bacterial regrowth after treatment. However, delay in development of resistance is a desirable success.

Keywords: *Litopenaeus vannamei*, *V. alginolyticus*, Choebdeh Abadan, $SPSP_W$ phage, Phage therapy

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Introduction

Farming pacific whiteleg shrimp (*Litopenaeus vannamei*) is an important economic activity. Aquaculture, as a seafood producing sector, is of vital importance supplying about 30 percent of seafood in the world in 2014 (FAO, 2014). Shrimp production, as the most favorite seafood with around 15 percent of overall value of internationally traded fishery products in 2012, was drastically reduced due to microbial contamination in 2016 (Stalin and Srinivasan, 2016). Extensive aquaculture is associated with environmental issues worldwide as a consequence of supersize development and high stocking density (Rebouças *et al.,* 2011). In fish and shellfish farming, infectious diseases caused by bacterial infections, are major inhibiting factors for aquaculture trade and production, that must be overcome (Oliveira *et al*. 2012). Viruses are commonly the main shrimp pathogens and reduce shrimp immunity, but superimposed bacterial infections accelerate mass mortality of shrimp (Xiaojing *et al.,* 2016). Sanitizing techniques (filters, ozone, UV, etc.) in marine hatcheries cannot provide a completely bacteria free environment and may cause microbial inequality leaving environmental niche exposed for the multiplication of opportunistic pathogens (Kalatzis *et al.*, 2016). *Vibrios* are diverse gram negative bacteria that are commonly found in tropical and temperate aquatic, estuarine and coastal sea habitats (Baker-Austin, 2015). Pathogenic species of *Vibrio*, as the main cause of disease issues in aquaculture (Intaraprasong *et al.,* 2009), under the stress conditions act as a secondary and opportunistic agent and might create 100 percent mortality (Selvin and Lipton, 2003). Several species of *Vibrio*, including *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus*, cause serious infections in farming aquatic animals (Rebouças *et al.,* 2011). Liu *et al*. (2004) reported up to 50 percent mortality in cultured *L. vannamei* shrimps, during 6 days of *Vibriosis* disease outbreak related to emergence of pathogenic gram negative, halophilic *V. alginolyticus* bacterium. As a mesophilic bacterium, *V. alginolyticus* is most pathogenic to breeding animal's and increases during summer and prevailing infections occur when immune performance of the target animal is compromised or the environment is polluted (Li *et al.,* 2019). Shakori and Rahimi (2016), in investigation of diversity and frequency of pathogenic *Vibrios* in shrimp culture ponds and water supply channels, in culture complex of Shahid Sanati, Gowater of Chabahar, reported that *V. alginolyticus* bacterium, pathogenic to shrimp larvae, was the dominant species with the greatest frequency. Application of antibiotics because of their usefulness against susceptible *vibrios* might increase the emergence of resistance in these microorganisms in a selective process, as a result of acquiring transferable antimicrobial resistance by mobile genetic elements

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and horizontal genetic transfer (WHO/FAO/OIE 2006, Rebouças *et al.*, 2011). Moghimi *et al*. (2014) investigated the resistance of two species of pathogenic bacteria, *V. harveyi* and *V. alginolyticus* isolated from three shrimp breeding centers in Bushehr Province, to streptomycin (S) and erythromycin (E). They reported that over consumption of these two antibiotics in human and livestock with the entrance of resistant bacteria to the two antibiotics via sewages and runoffs and transfer of resistant plasmid from bacteria with resistant plasmid to antibiotic, might be a resistant creating factor for them. Emergence of antibiotic resistant pathogens and inhibiting employment of antibiotics owing to detection of antibiotic remnants in shrimp tissues indicated a need for alternatives such as phage therapy as a promising and eco-friendly alternative for pathogenic bacteria growth inhibiting methods (Karunasagar *et al.*, 2007; Li *et al.*, 2019). Phage characteristics, such as their self-replicating nature which is based on possibility to apply once, may result in a cheaper dose application cost than chemotherapeutants (Carrias *et al.*, 2012). Also phage is self-limiting in association with the presence of the bacterial target (Kokkari *et al.*, 2018). Being in nature accessibility is at a relatively low cost (Zhang *et al.*, 2015) and complete consistent with the environment, performs selective removal and species specific bacteriolytic activity of the bacterial hosts without affecting natural microbiota (Kalatzis *et al.*, 2016). This makes them potential candidates for therapeutics and hindering of bacterial infections (Kokkari *et al.*, 2018; Li *et al.*, 2019). Therefore, the aim of this study was to isolate and characterize *Vibriophages* present in *L. vannamei* breeding and culture facilities and estuarine resources in south west of Iran, Choebdeh shrimp farming site, Abadan, Khuzestan Province, based on morphological structure, biological conditions and their potential applications in lysis of *Vibrio* bacteria.

Material and methods

Sampling

Sampling was carried out from estuarine sources, hatchery water and postlarvae culture tanks and *L. vannamei* shrimp culture ponds during April 2017 to November 2018, for isolation of *Vibrio* bacteria and their specific phages, in Choebdeh shrimp farming site, Abadan, Khuzestan Province, in south west of Iran. Water samples were obtained from 30 centimeters under the surface by sterile glasses with abrasive door (Otta *et al.*, 2001). 30 samples of water and 30 samples of sediment from mentioned sources were collected in sterile propylene containers (Vinod *et al.*, 2006; Chrisolite *et al.*, 2008). Physicochemical parameters, such as temperature, pH and salinity from sampling sites were measured in ranges of 26-27^{o}C, 8/5-8/8 and 20-30 PPT, respectively. Dilutions of water samples

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and water suspended sediment, was carried out serially using normal saline (0.9%), were streaked on plates of specific culture medium of *Vibrio* bacteria TCBS (Thiosulfate citrate bile salts sucrose agar, Biolife, Italia) and incubated in 30^{o}C for 18-36 hours. Also, TSB (tryptic soy broth, Biolife, Italia) culture medium, containing 1% of laboratory sodium chloride salt, was used for enrichment of supposed bacteria, before their transfer on TCBS culture medium. Yellow and green colonies were picked up and transferred into TSA (tryptic soy agar), containing 1% of laboratory sodium chloride salt and NA (nutrient agar, Merck, Germany) agar plates for further purification. Bacterial isolates were identified by different biochemical tests (Alsina and Blanch, 1994; Selvin and Lipton, 2003; Liu *et al.*, 2004) and kept in -80^{o}C by adding 15% glycerol for further study (Mateus *et al.*, 2014; Stalin and Srinivasan, 2016). Bacterial genomic DNA was extracted based on the method of phenol-chloroform-isoamyl alcohol with some modifications (Sambrook *et al.*, 1989). Bacterial isolates were identified using sequencing of whole gene 16S rRNA (1500 bp) with bacterial universal primer collection (27F:AGAGTTTGATCCTGGCTCAG) and (1492R:TACGGYTACCTTGTTACGACTT). Amplified PCR products were washed and purified by column kit method using SinaClon kit (SinaClon Company) and sequenced by Sanger method in ABI3730x1 apparatus. Acquired sequences, were aligned using Geneious IR9 software and analyzed by BLAST search in NCBI (national center for biotechnology information) center and higher gene sequencing identity of > 95% was determined (Stalin and Srinivasan, 2016).

Antibiotic susceptibility test and blood hemolysis

Antibiotics susceptibility was investigated based on Stalin and Srinivasan (2016), i.e., antibacterial disc diffusion method on Muller-Hinton's agar plates. Bacterial samples were tested against available commercial antibiotic discs, such as Ampicillin (AM10), Azithromycin (AZM15), Nalidixic acid (NA30), Sulfamethoxazole (SXT), Vancomycin (V30), Erythromycin (E15) and Penicillin (P10). Zone of inhibition around the discs was measured and compared with interpreted diagram showing the type of bacterial resistant (Bauer *et al.*, 1966; Marhual *et al.*, 2012; Stalin and Srinivasan 2016). In order to evaluate blood hemolysis ability and confirm pathogenic nature of the bacterial species, *V. alginolyticus*, (Alagappan *et al.*, 2013), human blood agar plates were used.

Isolation, purification and determining of phage concentration

In order to isolate phages, the method of Karunasagar *et al*. (2007) with some modifications was applied. Tryptic Soy Broth 2x (TSB2X) culture medium

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containing 1-2% of laboratory sodium chloride salt, was inoculated with the bacterial host (*V. alginolyticus*), for the enrichment of water and sediment, samples containing supposed phage (s) were used. After incubation and high centrifugation, the supernatant was passed throw 0.45 and 0.22µm filters. Phage existence was detected by inoculation of 25µL of filtered supernatant on bacterial layers in solid culture medium, supplemented with 1% NaCl (TSAS). Clear plaques on the culture medium, showing the phage lytic activity, were picked up and inoculated into the fresh culture medium (TSBS) for the phage enrichment and purification (Karunasagar *et al.,* 2007; Chrisolite *et al.,* 2008). For determining the phage concentration, the agar overlay method was used (Chrisolite *et al.,* 2008). The phage containing supernatants were diluted up to 10^{-5} using standard SM sterile buffer (Tris HCl 0.05M, pH 7.4, NaCl 0.1M, $MgSO_4$ 10 mM, gelatin 1% w/v) then incubated with the index isolate (*V. alginolyticus*), suspended in low percent agar (0.7%) and finally plated on solid agar (Liu *et al.,* 2014). After 6-16 hours of incubation in 26±2^{o}C, the phage plaques were numerated and expressed as $PFUmL^{-1}$ (Muniain-Mujika *et al.,* 2000; Vinod *et al.,* 2006; Liu *et al.,* 2014).

Transmission electron microscopy (TEM)

The lytic phage isolated in this study, was classified according to Oakey and Owens (2000) and Ackermann (2006) based on morphological characteristics, such as head shape, neck region, to have a specific tail or not and tail sheath. After purification of the phage suspension (11×10^9 $PFUmL^{-1}$), one drop of the suspension was placed on carbon coated copper grid for 1 min, allowing the phage to be absorbed by the grid. The grid was negatively stained with 2% Uranyl acetate for 60 seconds. Then the grids were dried with filter paper in order to remove the extra stain. Electronic micrographs were taken using Philips TEM cm300 (Philips, Netherlands) at 200 kV accelerating voltage and 40000 X magnification (Jun *et al.,* 2014; Liu *et al.,* 2014).

One-step growth experiment

The one-step growth experiment was carried out based on Stalin and Srinivasan (2016) with some modifications. 500 µL of fresh cultured *V. alginolyticus* was incubated with 500 µl of 10^{-1} the $SPSP_W$ phage dilution for 5 minutes at ambient temperature (25±1^{o}C). After high centrifugation, the supernatant was discarded and the achieved sediment resuspended in nutrient broth (NBS) culture medium supplemented with 1.5% NaCl (Lin *et al.,* 2012). This mixture was incubated at 30^{o}C at 80 rpm on shaker to allow phage multiplicity infection. 1 mL samples of the mixture were obtained at 600 nm in 10 min intervals up to 120 min and were immediately centrifuged at high speed. The filtered (0.45 µm)

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supernatant was serially diluted (10^{-5}) in SM buffer. The phage concentration in suspension was assessed by agar overlay method to determine the rate of plaque formation (PFU), latent periods and burst sizes (Liu *et al.,* 2014; Mateus *et al.,* 2014; Zhang *et al.,* 2015; Stalin and Srinivasan, 2016).

Phage thermal, salinity and pH stability test

The $SPSP_W$ phage enrichment was carried out by mixing 1 mL of each of the *V. alginolyticus* and the phage at first dilution (10^{-1}) and inoculate this mixture into 20 mL of N.B culture medium containing 2% NaCl. After overnight incubation at 28±2°C at 80 rpm, 1900×10^6 $PFUmL^{-1}$ of filtered supernatant was formed. The $SPSP_W$ phage stability at different temperature, salinity and pH was examined by pre-incubating for 1 h based on Stalin and Srinivasan (2016). After incubation, the phage concentration was tittered/counted by double layer agar plate method (Jun *et al.,* 2014).

In vitro phage therapy assay

In order to evaluate bacteriolytic activity of the $SPSP_W$ phage against the bacterial species, *V. alginolyticus*, spectrophotometric method based on formation of turbidity due to the bacterial growth was used (Jeon *et al.,* 2016). In order to obtain a multiplicity of infection (MOI) of 73, 30 µL of the bacterial isolate at early exponential phase (1.5×10^8 $CFUmL^{-1}$) at optical density value of 0.1 at 600 nm and 300 µL of the phage suspension of about 11×10^9 $PFUmL^{-1}$ was inoculated into sterilized glass Erlenmeyers with 30 mL of NB medium containing 1.5% of NaCl as a treatment group and incubated at 27±1 °C without shaking in dark condition. The *V. alginolyticus* that wasn't inoculated with the $SPSP_W$ phage (MOI: 0), was applied as a control group. The absorbency values at 600 nm, at 0, 12 and 24 h after the bacterial incubation, were determined (Mateus *et al.,* 2014; Jeon *et al.,* 2016).

Statistical analysis

The statistical analysis was performed using SPSS software, version 24. Existence of significant differences between the treatments with the phage was compared with the control group (just bacterium) was assessed by one-way analysis of variance (ANOVA) test with the Duncan's multiple range test and least significance difference (LSD) to compare individual means. The mean values with $p<0.05$ were considered to be significantly different (Stalin and Srinivasan, 2016).

Results

Biochemical characteristics and 16S rRNA genes sequence analysis

As shown in figure 1, the colour of *V. alginolyticus* colonies on TCBS culture medium was yellow. The *V. alginolyticus* isolates were gram negative with short rod structure and tolerant to different salinities and temperatures (Table 1).

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Table 1: Biochemical characteristics of *V. alginolyticus* isolated from *L. vannamei* shrimp from culture farms in comparison with the *V. alginolyticus* reference isolate ATCC17749 used by Liu et al. (2004).

Test	*V. alginolyticus*	ATCC17749
Gram staining	-	-
Cell structure	rod	rod
Growth on TCBS	+ (yellow)	+ (yellow)
Oxidase	+	+
Lysine decarboxylase	+	+
Ornithine decarboxylase	+	-
Simmons citrate	v	-
Indole	+	+
Arginine dehydrolase	v	-
Gas from glucose	-	-
Lactose fermentation	-	-
H_2S production	-	-
NaCl tolerance		
TSA 1%	+	+
TSA 8%	+	+
TSA 10%	+	+
Thermal tolerance		
4°C	-	-
40°C	+	+
50°C	v	-

+: positive; -: negative; v: variable.

The bacterial isolates identified by physicochemical parameters mentioned in Table 1, were confirmed as *V. alginolyticus* species by 16S rRNA gene sequencing and compared with the acquired sequences by others in NCBI. The acquired whole sequence of *V. alginolyticus* isolate showed in figure 1,

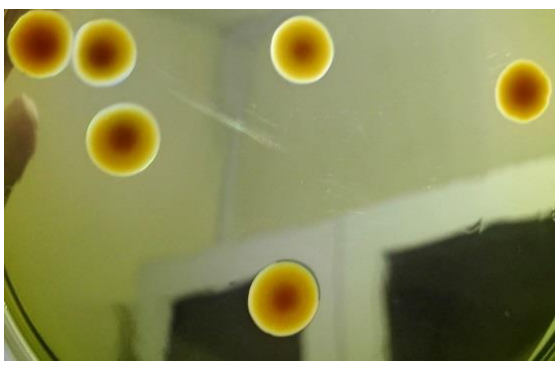


Figure 1: The colonies of *V. alginolyticus* isolated in this study on TCBS.

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was as:

"AAGTGGTAAGCGTCCCCCCGAAGGTTAAACTACCTACTTCTTTTGCAGCCCACTCC
CATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCATTCT
GATCCACGATTACTAGCGATTCCAACTTCATGGAGTCGAGTTGCAGACTCCAATCC
GGACTACGACGCATTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCCGCCCTCTGTA
TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGT
CGTCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTAC
TCGCTGGCAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTC
ACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCAC
CAATCCATCTCTGGAAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTT
GCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTG
AGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCTACTTAACGCGTTAGCTCCGAA
AGCCACGGCTCAAGGCCACAACCTCCAAGTAGACATCGTTTACGGCGTGGACTACC
AGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCATCTGAGTGTCAGTATCTGTC
CAGGGGGCCGCCTTCGCCACCGGTATTCCTTCAGATCTCTACGCATTTCACCGCTAC
ACCTGAAATTCTACCCCCCTCTACAGTACTCTAGTCTGCCAGTTTCAAATGCTATTC
CGAGGTTGAGCCCCGGGCTTTCACATCTGACTTAACAAACCACCTGCATGCGCTTT
ACGCCCAGTAATTCCGATTAACGCTCGCACCCTCCGTATTACCGCGGCTGCTGGCA
CGGAGTTAGCCGGTGCTTCTTCTGTCGCTAACGTCAAACGATCACGCTATTAACGT
ACCCCCCTTCCTCACGACTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACAC
GCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCC
CGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGATCATCCTCTCAGACCA
GCTAGGGATCGTCGCCTTGGTGAGCCCTTACCTCACCAACTAGCTAATCCCACCTA
GGCATATCCTGACGCGAGAGGCCCGAAGGTCCCCCTCTTTGGCCCGTAGGCATCAT
GCGGTATTAGCCATCGTTTCCAATGGTTATCCCCCACATCAGGGCAATTTCCTAGGC
ATTACTCACCCGTCCGCCGCTCGACGCCGTATCGTTCCCCGAAGGTTCAGATAACT
CGTTCCGCTCGACTGCATG".

Based on the diameter of growth halo (mm) formed around the antibiogram disc on Muller-Hinton's agar plate, *V. alginolyticus* isolates as well as *V. harveyi*, as a standard sample, after incubation for 18-24 h at 37^{o}C, were resistant to ampicillin and penicillin, intermediate to erythromycin, but *V. harveyi* isolate was resistant. For vancomycin there was a sensitive and intermediate state and *V. harveyi* was intermediate. Sensitive attitude was observed for nalidixic acid, sulfamethoxazole and azithromycin in both *V. alginolyticus* isolates and *V. harveyi*. Evaluation of haemolysis ability of *V. alginolyticus* isolates, using human blood on blood agar plates indicated that these bacterial isolates were Beta haemolytic and pathogenic. Application of agar overlay method by mixing of *V. alginolyticus* isolates with $SPSP_W$ phage filtrate resulted the appearance of the phage plaques (Fig. 2).

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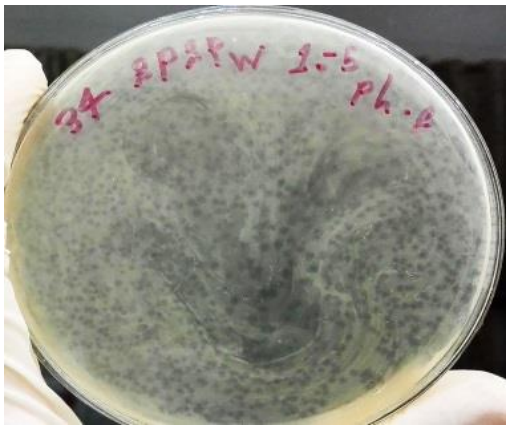


Figure 2: The phage plaques formed with *V. alginolyticus* infected with the enriched phage $SPSP_W$ by double-layer agar plate.

Transmission electron microscopy characterization of phage morphology

Morphological investigation of $SPSP_W$ phage, isolated from sediments around paddle wheel aerators of *L. vannamei* from shrimp culture ponds, by TEM analysis showed that this phage have a hexagonal head and very short tail with distinguishable tail fibers belonging to *Podoviridae* family (Fig. 3).

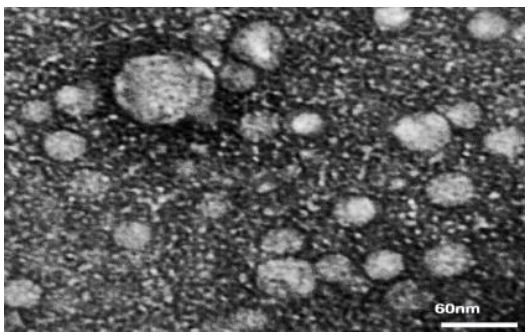


Figure 3: TEM image of negatively stained *V. alginolyticus Podovirus* phage.

Determination of phage survival

In evaluation of $SPSP_W$ phage stability based on the phage plaques formation at 30-65^oC for 1 h, the most and least number of plaques, i.e. 1200 and 0, were observed at 35 and 65^oC, respectively. There were 8 plaques at 61^oC (Fig. 4A). The salinity stability test showed that $SPSP_W$ phage had the most and least number of plaques, i.e. 1010 and 350 at 3 and 10% of salinity, respectively (Fig. 4B), also the pH stability test indicated that isolated phage had the highest and lowest number of plaques, i.e. 650 and 0 at pH 8 and pH 3, respectively. At pH 4 and pH 11, 125 and 200 plaques were numerated, respectively. In spite of expectation in reduction of plaque numbers at pH 12, the number of plaques increased to 420 (Fig. 4C). In evaluation of one-step growth curve for $SPSP_W$ phage for 2 h, we had increase in plaque numbers at 1 h then reduction and after that increase in plaques with a moderate trend. The short latent period was about 30 min and rise period was up to 60 min, then reduction until 70 min and re-increase in the phage plaques was detected. The highest burst sizes 191×10^6 $PFUmL^{-1}$ and 205×10^6 $PFUmL^{-1}$ were observed at 60 min and 120 min after phage inoculation, respectively (Fig. 5).

In vitro phage therapy assay

After 8 h of $SPSP_W$ phage and *V. alginolyticus* bacterial isolate inoculation and incubation at 27±1^oC in the phage therapy assay, we observed turbidity due to bacterial growth in the bacterial control, but the bacterium growth wasn't allowed by the phage in the treatment category and bacterial growth and turbidity was observed after 11 h of incubation.

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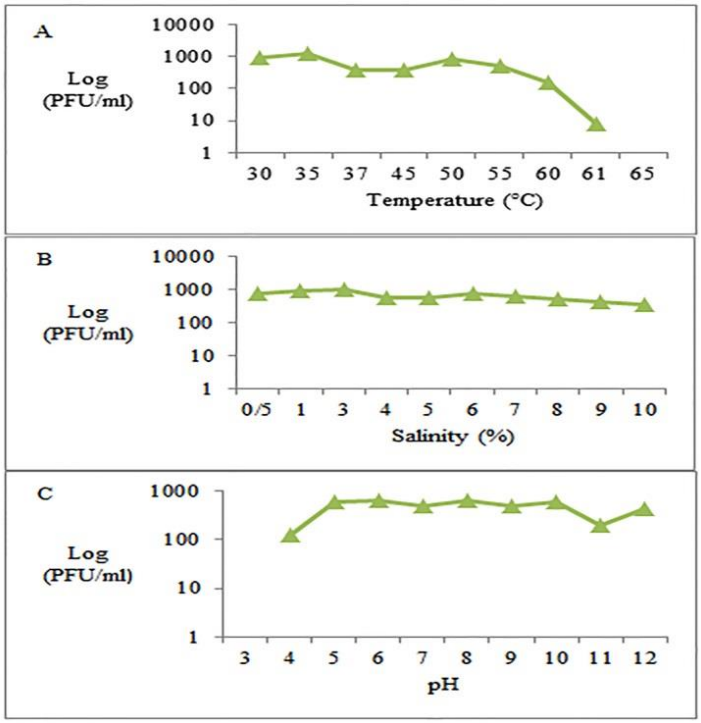


Figure 4: Stability test for the survival of *V. alginolyticus* phage: (A) temperature; (B) salinity and (C) pH values.

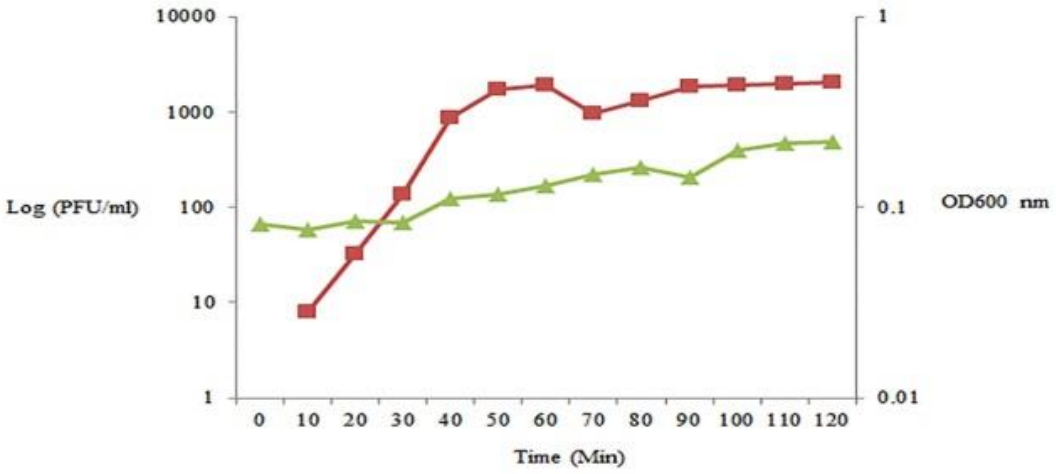


Figure 5: Diagram of one-step growth pattern for *V. alginolyticus Podovirus* phage along with absorbency values (OD_{600}) for *V. alginolyticus* growth.

Table 2 shows the data related to the mean of absorbency values (OD_{600}) in the time range of 0, 12 and 24 h after inoculation that is indicating the target bacterium growth in control category (without phage) and treatment category (with phage). Analysis of the mean of differences between control and treatment categories indicated that there is a significant difference (p<0.05), between these two categories (Table 3).

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Table 2: The mean of absorbency values in challenging categories of phage therapy.

Time (h)	Control	Treatment*
	V. alginolyticus	**$SPSP_W$ × *V. alginolyticus***
0	0	0
12	1.182	0.321
24	1.257	1.034

*Mean of three replicates

Table 3: Analysis of the mean of differences between two categories of control and treatment.

Category	Mean	Groups	
Control	0.813	A	
Treatment	0.452		B

Discussion

Generally, *Vibriosis* in penaeid shrimps is diagnosed as a secondary infection that is affected by factors such as stress, unusual and detrimental alterations in environment and high numbers of potential pathogenic bacteria (Liu *et al.*, 2004). Alterations in environmental factors, such as salinity and pH are able to change the virulence of *V. harveyi* and *V. alginolyticus*. Also, non-application of appropriate stocking densities resulted in decreased immunity and resistance to *V. alginolyticus* and WSSV (Lin *et al.*, 2015). *V. alginolyticus* is one of the most common bacterial species in sea aquaculture hatcheries and several reports indicating the interference of this bacterium in disease outbreaks are exist (Kokkari *et al.*, 2018). In the view of biochemical characteristics, *V. alginolyticus* bacterial isolates investigated in this study, isolated from hatchery and the shrimp (*L. vannamei*) culture farms, showed various responses as positive and negative in relation to methyl red and voges proskauer tests. They had a short rod shape and based on colony colour on TCBS culture medium were yellow. This bacterial isolates were indole positive, variable to arginine and Simmons citrate, ornithine positive and also the growth ability at 0.5% NaCl and 1-10% NaCl was positive. In some of the biochemical tests, *V. alginolyticus* isolates studied in this study, have different results than the *V. alginolyticus* strains studied by Alsina and Blanch (1994), Selvin and Lipton (2003). This subject shows the difference in genotypic characteristics (Hernandez and Olmos, 2004). The *V. alginolyticus*, which were isolated and purified in our study, had positive haemolytic activity type β. According to the Alagappan *et al.* (2013) most of pathogenic *Vibrio* sp have virulence characteristic such as haemolytic activity. Having haemolytic activity is a differentiation index for pathogenic and nonpathogenic bacteria (Alagappan *et al.*, 2013); this principle indicates the pathogenic nature of *V. alginolyticus* bacterium isolated in this study. The key role of 16S rRNA gene sequencing performed is confirmed in bacterial identification in this study, because of more potency and accuracy than phenotypic evaluation and capability of identification of rare bacteria that are

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not cultivable and have an unusual phenotypic profile (Stalin and Srinivasan, 2016). In the present study, evaluation of susceptibility of *V. alginolyticus* isolates to traditional antibiotics using antibacterial disc diffusion method (Bauer *et al.,* 1966) on Muller-Hinton's agar plate showed that this bacterial isolate was sensitive to vancomycin, nalidixic acid, sulfamethoxazole and azithromycin. The zone of inhibition for vancomycin, nalidixic acid, sulfamethoxazole and azithromycin was 12, 25.8, 23.4 and 22.9, respectively. In this study, the obtained zone of inhibition for nalidixic acid was completely consistent with NCCLS (1998) that is indicating the sensitivity of this bacterial isolate to the mentioned antibiotic (Bauer *et al.*, 1966, Stalin and Srinivasan, 2016). The bacterial isolate was completely resistant to penicillin and ampicillin and generally was not affected by the two types of antibiotics. Our results were in line with findings of Stalin and Srinivasan (2016) and Marhual *et al*. (2012) in *V. parahaemolyticus* and *V. alginolyticus*, also were in line with Selvin and Lipton (2003) and Rebouças *et al*. (2011) in *V. alginolyticus* that showed their resistance to ampicillin. Also *Vibrio* bacteria studied by Otta *et al*. (2001) were resistant to amoxycillin and ampicillin. The morphological survey of $SPSP_W$ phage using TEM at 40000 X (60 nm) showed that this phage having an icosahedral head and very short tail, belongs to *Podoviridae* family (Ackermann, 2006); and isolation of this phage in relation to *V. alginolyticus* is shown by many studies (Lin *et al.,* 2012; Zhang *et al.,* 2014; Kokkari *et al.,* 2018). The *Podovirus* family is characterized by phages with icosahedral and isomorphous head and a non-contractile tail (Lin *et al.,* 2012). The $SPSP_W$ phage had an excellent function in terms of biodegradation of isolated *V. alginolyticus* in the present study. In this study, clear plaques obtained by application of double layer agar method, represent the lytic ability of $SPSP_W$ phage against *V. alginolyticus* bacterial cells (Liu *et al.,* 2014; Mateus *et al.,* 2014; Zhang *et al.,* 2015; Stalin and Srinivasan, 2016). From the aspect of stability in different environmental conditions, temperature, salinity and pH had great importance in relation to control of the pathogenic *Vibrio* species in shrimp culture farms (Chen *et al.,* 2015). $SPSP_W$ phage had an acceptable stability at 4^oC even after 6 months. This phage had an optimal growth in up to 50^oC that was consistent with findings of Kokkari *et al*. (2018) in *Podoviridae* phage specific to *V. alginolyticus*. In this study, the identified phage formed the plaques at 61 ^oC that was different from findings of Stalin and Srinivasan (2016) in *V. parahaemolyticus* by *Myovirus* phage that didn't observe any phage plaque at 60^oC. The phage in this study had a good stability at 0.5-10% as well as pH 4-12 that is consistent with the growth range of most *Vibrio* bacteria (Alsina and Blanch, 1994; Karunasagar *et al.,* 1996). $SPSP_W$ phage possessed of

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great function at pH 8-10, especially at pH 8 in terms of bacterial clearance and phage persistence after 36 h of overlaying phage and bacterium. These results are similar with the results obtained by Li *et al*. (2019) that showed the optimal pH of 7-9 for *V. alginolyticus* infected by *Podovirus*, but in terms of thermal stability the phage isolated in the present study had higher stability. Survivability at changing environment is one of the key factors in success of the phage mediated control of *Vibrio* bacteria (Mateus *et al.*, 2014). This stability is very beneficial for the phage scale production and long-term storage (Kokkari *et al.*, 2018). In evaluation of one-step growth curve of $SPSP_W$ phage for 2 h, the latent period obtained in our study was less than the investigated phages by Mateus *et al*. (2014) with *V. parahaemolyticus* bacterium that acquired the time period of between 40 to 120 min for initiation of exponential phase, or the same phage burst size in 80 min in study of Li *et al*. (2019). Evaluation of phage therapy with $SPSP_W$ phage and *V. alginolyticus* bacterial isolate indicated that bacterial growth in treatment group was reduced remarkably and only after 11 h of inoculation, turbidity caused by bacterium growth was observed ophthalmologically. The results of in vitro evaluation of $SPSP_W$ phage for control of *V. alginolyticus* isolate indicated that the *Podovirus* phage isolated in this study, was significantly ($p< 0.05$) capable to control *V. alginolyticus* population (Stalin and Srinivasan, 2016, Kokkari *et al.*, 2018). In real aquaculture experience, the results of phage therapy could completely be different from the results obtained at laboratory works. The differences between bacterial isolates, their concentration or even phage behavior in a non-axenic or Gnotobiotic might affect the outcome of the page therapy. The high diversity between pathogenic bacterial agents indicates that achieving a general phage therapy solution is difficult. In turn the phages isolation against local pathogenic agents could be a feasible and low-cost alternative (Kokkari *et al.*, 2018).

Phage therapy with single phage suspension doesn't inhibit the regrowth of the target bacterium after treatment and finally the host bacteria will develop resistance against the phage. However, delay in resistance developing by bacteria is a desirable success (Mateus *et al.*, 2014). In cases that lytic efficiency of a phage is low, this phage must be used in combination with other lytic phages to form a robust phage cocktail (Kokkari *et al.*, 2018).

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