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GENETIC PROFILING OF VIBRIO HARVEYI BACTERIOPHAGES FROM WATER SAMPLES USING DEGENERATE PRIMED RANDOMLY AMPLIFIED POLYMORPHIC DNA FINGERPRINTING

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ABSTRACT

Vibrio harveyi poses a significant threat to shrimp aquaculture. In this study, we isolated seventy-six bacteriophages infecting luminescent *V. harveyi* from 194 water samples sourced from shrimp hatcheries along the South East coast and Andaman island of India. Utilizing Degenerate Primed Randomly Amplified Polymorphic DNA (DP-RAPD) fingerprinting, we investigated the genetic relatedness among these bacteriophages. The analysis, based on Dice coefficient and subsequent construction of a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA), unveiled 12 distinct clusters. Transmission electron microscopy observations were conducted on one randomly chosen phage from each major cluster, revealing that eleven exhibited an icosahedral head (46-115 nm) with a long non-contractile tail (132-329 nm), classifying them under the *Siphoviridae* family. Meanwhile, two phages displayed a short tail (15-27 nm), placing them in the *Podoviridae* family. Phylogenetic analysis of the phages using DP-RAPD fingerprinting demonstrated partial correlation with the host's phenotypic characteristics, particularly in sucrose fermentation and source of isolation. However, phages infecting *V. harveyi*, despite belonging to different families, did not cluster together in the DP-

PCR cluster analysis. This study underscores the genetic diversity among phages infecting the same host, particularly in light of phenotypic variations, as revealed by DP-RAPD.

KEYWORDS: Bacteriophages, DP-RAPD, *Podoviridae*, *Siphoviridae*, *Vibrio harveyi.*

INTRODUCTION

Vibrio harveyi stands out as a significant pathogen causing widespread mass mortalities among penaeid shrimp larvae in hatcheries, leading to substantial economic losses (Karunasagar et al., 1994; Austin and Zhang, 2006). Despite efforts to control the luminous bacterial disease (LBD) using antibiotics, the efficacy in field conditions has been unsatisfactory, accompanied by the emergence of resistant strains (Baticados et al., 1990; Moriarty, 1999; Karunasagar et al., 1994).

Bacteriophages, or phages, are viruses that selectively infect bacteria, either lysing them or remaining dormant as prophages. Found ubiquitously in environments colonized by their bacterial hosts, bacteriophages have been explored as an alternative to antibiotics in treating bacterial infections (Defoirdt et al., 2011). The concept of phage therapy in aquaculture has gained attention from researchers (Nakai and Park, 2002; Vinod et al., 2006; Karunasagar et al., 2007).

Traditional bacteriophage taxonomy relies on factors such as shape, size, morphology, serology, and genetic content. The International Committee on Taxonomy of Viruses classifies bacteriophages primarily based on morphology and nucleic acid into the order Caudovirales, comprising families such as Myoviridae, *Siphoviridae*, and *Podoviridae* (Ackermann, 2007, 2009). While classical methods involve DNA analysis, protein analysis, and electron microscopy, modern approaches like pulsed-field gel electrophoresis (PFGE) and restriction digestion provide initial classification, with PCR, shotgun cloning, and sequence analysis offering more comprehensive insights (Carlson, 2005). Recent advancements, such as fluorescence-labeled restriction fragment length polymorphism (fRFLP), have revealed that genetic relatedness among phages does not always align with their host range (Merabishvili et al., 2007).

In the context of genetic diversity assessment, techniques like RAPD-PCR and low stringency PCR amplification using degenerate primers (DP-RAPD) have been applied to vibriophages and phages infecting various bacteria (Comeau et al., 2006; Shivu et al., 2007; Dini and De Urraza, 2010; Li et al., 2010). Our study, building upon previous work characterizing bacteriophages of the *Siphoviridae* and *Podoviridae* families, focuses on isolates from shrimp hatcheries in South India. Through DP-PCR, cluster analysis, and transmission electron microscopy, we aim to differentiate these bacteriophages based on their lytic spectrum and the phenotypic nature of their hosts. This investigation is crucial in evaluating the potential of these phages as biocontrol agents for the

luminous bacterial disease in larval tiger shrimp.

MATERIALS AND METHODS

Bacteriophagesof*Vibrio harveyi*

This study utilized seventy-six bacteriophages, specifically designated as *Vibrio harveyi* phage (VHP 01 to VHP 76) according to the nomenclature by our research team (Chrisolite et al., 2008). The propagation of these phages was carried out in broth cultures of their respective hosts, following the methodology outlined by Carlson in 2005. In brief, *V. harveyi* hosts (n=27) were sub-cultured in 5 ml peptone yeast extract sea salt (PYSS) broth, containing 5 g L-1 peptone and 3 g L-1 yeast extract dissolved in Macleod's artificial sea salt water (HiMedia, India). One milliliter culture was inoculated into 50 ml of PYSS broth and incubated at 30 °C for 4-6 hours in an orbital shaker at 120 rpm, achieving a cell density of approximately 10^8 colony forming units per ml (cfu ml-1).

Subsequently, the bacterial cultures were infected with one ml of the respective phage stock and further incubated for about 6-8 hours at 30 °C. Phage lysate preparation involved centrifugation of the phage-infected broth twice at 10,400× g for 15 minutes at 4 °C. The resulting supernatant was filtered through a 0.45-µm filter (Millipore, USA). Bacteriophages in the filtrate were concentrated through ultracentrifugation at 200,000 x g for 2 hours at 4°C, utilizing an SW-41 swinging-bucket rotor (Beckman, CA). The phage pellet obtained was then resuspended in sterile phage buffer (0.05M Tris-HCl, 0.02M MgSO4, pH 7.5; Ghosh et al., 1989).

To remove nucleic acid residues of the host bacteria, the suspension underwent treatment with DNase I (1 µg ml-1) and RNase A (100 µg ml-1, Merck, India). The resulting phage concentrate was purified by ultracentrifugation at 490,000 x g for 18 hours at 20°C, using a discontinuous Cesium Chloride (CsCl; SRL, India) density gradient (d = 1.7, 1.5, 1.3 g ml-1) prepared in phage buffer (Sambrook and Russell, 2001). The band containing phage particles was carefully drawn from the centrifuge tube using a sterile needle fitted to a syringe. CsCl was subsequently removed through dialysis at 4°C against phage buffer.

ExtractionofphageNucleicAcid

The extraction of total nucleic acid from bacteriophages followed a previously outlined protocol (Santos, 1991) with slight modifications. In brief, 1 ml of CsCl-purified phage suspension, pre-treated with DNase I and RNase A, was combined with 0.5 ml of TESS buffer (0.1M Tris-HCl pH 8, 0.05M EDTA pH 8, 0.3% SDS, and 0.1 M NaCl) and incubated at 56 °C for 5 minutes. Subsequently, 100µg of Proteinase K (Finnzymes, Finland) was introduced, followed by an additional incubation at 56 °C for 30 minutes. The mixture underwent extraction twice using a

phenol: chloroform: isoamyl alcohol solution (25: 24: 1). The resulting aqueous phase was transferred to a separate tube and combined with 0.54 volumes of isopropanol to induce nucleic acid precipitation. The ensuing pellet was washed with 70% alcohol, briefly air-dried, and then re-suspended in 50 µl of sterile Milli-Q water.

DP-RAPDGenomicfingerprintingandclusteranalysis

The degenerate-primed random amplification of polymorphic DNA (DP-RAPD) was conducted following a previously established method (Comeau et al., 2004) employing the R10D primer sequence 5'-GTCASSWSSW-3', where S and W represent G/C and A/T, respectively. To optimize the DP-RAPD, reactions were performed at three different concentrations (50 pM, 100 pM, and 200 pM) of the R10D primer, incorporating 3 mM magnesium chloride instead of the standard 1.5 mM concentration in commercial Taq buffer, and utilizing a gradient of annealing temperatures ranging from 35 to 45°C.

The optimized PCR reactions were carried out in 25 µL reaction mixes, including 2.5 µL of 10 × Taq buffer with 200 µM of each deoxynucleoside triphosphate, 3 mM magnesium chloride, 200 pM R10D primer, 1.5 U of Taq DNA polymerase (Invitrogen, USA), and 40 ng of template DNA. PCR was conducted in a thermocycler (Eppendorf, Germany) with the following thermal cycling conditions: initial denaturation at 95°C for 1.5 min, followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 40°C for 3 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

DP-RAPD PCR products and 100 bp ladders (Invitrogen, USA) underwent electrophoresis through 1.5% agarose (Himedia, India) in 0.5 × TBE buffer (45 mM Tris–borate, 1mM EDTA; pH 8.0) at 90 V for 1.5 h. The gels were stained with ethidium bromide (0.5 mg mL-1) and captured using the Quantity One electrophoresis analysis system (Bio-Rad, CA, USA). To minimize gel-to-gel banding pattern variability, gel images were digitally normalized to a single DNA marker. Cluster analysis was conducted using Molecular Analyst software – Fingerprinting II (Version 3.0, Bio-Rad, CA), with the similarity matrix calculated based on the Dice coefficient. The corresponding dendrogram was generated using the unweighted pair group method with arithmetic averages (UPGMA).

Electronmicrocopy

A 10µL suspension of purified phages was applied onto 200 mesh carbon-coated copper grids and subjected to staining with 2% potassium phosphotungstate (pH 7.2) for 20 seconds. Any excess stain was promptly eliminated by placing the grids on blotting paper. Subsequent examination of the grids was performed using a Transmission Electron Microscope (Tecnai G2 Spirit Bio-Twin,

Eindhoven, The Netherlands).

Resultsanddiscussion

Bacteriophages are considered to be important components of natural microbialecosystems.Theyareubiquitousandmostabundantbiologicalentitiesonearthandplaykeyroles in regulating the microbial balance in every ecosystem through the lysis of bacterialcells or through horizontal gene transfer (Fuhrman, 1999, Wommack and Colwell, 2000,Williamsonetal.2003).Despitethehighabundanceofphageswithinaquaticenvironments,application ofmoleculargeneticstoolsandmicroscopytostudyviralecologyoftenrequiresthatvirusparticlesareextracted andconcentrated.

Inthis study, the purificationof phage particlesisolatedfrom shrimp hatcheryenvironment was done by CsCl gradient ultracentrifugation. The purified phage particleswerefoundasfaintwhitishbandsatCsCldensitynearto1.5gmmL-1.Knowntailedphages,belonging to all three families and infecting many different Gram negative and Gram-positivebacteria,areallcomposedofapproximatelyequalamountsofproteinandDNA,givingthema buoyant density in CsCl between 1.45 and 1.52 gm mL-1 (average 1.49; lipid-containingphagesaverage1.3gmL-1(Fraenkel-Conrat,1985).Thetiterofphagesinthepurifiedstockwasestimatedtobeintherange of2.8×1012to8×1012pfumL-1.

The DNA extraction from the phage concentrates yielded 0.2 to 1µg of DNA.Appropriate dilutions of DNA were made to get uniform template concentration (40 ng).However, recently it was reported that phage suspensions are also suitable to generatereproducible RAPD profiles, bypassing the need for isolating DNA, suggesting that RAPD-PCRcouldbeaneasytechniquetoassessthegeneticdiversityamongphages(Gutierrezetal.2011).

GenomicfingerprintingofphagesbyDP-RAPD

The DP-RAPD profiles of 76 phages exhibited a band count ranging from 1 to 12, with amplicon sizes spanning 126 to 1800 bp. Among these, 56 phages generated 2-6 bands, 15 phages produced 8-12 bands, and five phages produced a solitary band, approximately 537 bp in size. A previous study (Comeau et al., 2004) employing the same primer for algal viruses and bacteriophages with diverse genome sizes and virus families reported that the fingerprints generally comprised fewer bands compared to larger algal virus fingerprints. However, they noted a moderate linear relationship between genome size and the number of bands. The study further emphasized that closely related viruses within the same Phycodnaviridae family, infecting distinct hosts like Micromonas pusilla and Chlorella-like algae, exhibited similar yet distinct

patterns.

Amid various PCR-based DNA fingerprinting methods for molecular typing of bacterial viruses, RAPD utilizes arbitrary primers to detect changes in DNA sequence, offering a relatively fast and easy approach for molecular epidemiological typing (Williams et al., 1990; Gutierrez et al., 2011). Phages infecting closely related bacterial hosts typically exhibit little or no nucleotide sequence similarity, while those infecting the same bacterial host tend to be more similar (Hatfull, 2008).

To validate the utility of the DP-RAPD-PCR protocol for phage typing, experiments were conducted on phages infecting *V. harveyi* from the same source, testing various conditions to generate reproducible RAPD patterns and assess the method's discriminatory power. The R10 D primer was tested at three concentrations (50, 100, and 200 pM), with lower concentrations yielding less defined bands. Increased magnesium chloride concentration resulted in clearer band patterns, as Mg2+ ions stimulate the action of DNA polymerase (Pomp and Medrano, 1991). Optimal results were achieved with 3 mM magnesium and 200 pM primer concentration at an annealing temperature of 40°C.

Cluster analysis of DP-RAPD profiles (Fig.1) categorized the 76 phages into 12 groups at a 34% hierarchical level. Morphological observations of one randomly selected phage from each cluster using TEM (Table 1, Fig. 2) revealed that eleven belonged to the *Siphoviridae* family, displaying a head size of 46-115 nm with a long, non-contractile tail of 132-172 × 7-13 nm. Two phages belonged to the *Podoviridae* family, featuring a head (72-77 nm) with a short tail (15-27 nm). The clustering somewhat aligned with the phenotypic nature of the host and the source of isolation.

Notably, clusters 2, 7, 9, 10, 11, and 12A comprised only 2-3 phages each, while cluster 12B grouped five phages, including two podoviruses. The observed clusters correlated to some extent with the host's phenotypic characteristics and isolation source. However, the DP-PCR cluster analysis did not group phages specifically infecting *V. harveyi* or those belonging to different families together. The study demonstrated that, despite certain correlations, the genetic diversity of phages infecting the same host, with respect to phenotypic differences, was revealed by DP-RAPD. Among the 76 phages, 52 were able to lyse other closely related vibrios like V. alginolyticus, V. parahaemolyticus, and V. logei, indicating a broad lytic spectrum.

Fifteen phages were specific to *V. harveyi*, yet they did not cluster together in the DP-PCR analysis. This observation aligns with previous findings suggesting that genetically distinct

phages infecting the same host tend to cluster together based on RAPD fingerprinting. The study provides insights into the genetic diversity and potential biocontrol applications of phages infecting *V. harveyi* in the context of shrimp hatchery management.

CONCLUSIONS

In conclusion, the application of Degenerate Primed Randomly Amplified Polymorphic DNA (DP-RAPD) profiling proved to be a valuable and effective method for assessing the genetic diversity among bacteriophages infecting *Vibrio harveyi*. The phylogenetic analysis based on DP-RAPD fingerprints successfully categorized the 76 *V. harveyi* phages into 12 distinct clusters. Remarkably, these clusters exhibited a correlation with the phenotypic characteristics of the host bacteria, particularly in terms of sucrose fermentation and the source of isolation. This finding underscores the utility of DP-RAPD as a molecular tool capable of revealing the genetic relationships among phages and their hosts, providing valuable insights into the intricate dynamics of bacteriophage populations. The study contributes to our understanding of the genetic diversity of *V. harveyi* phages, which is crucial for potential applications in biocontrol strategies, especially in the context of shrimp hatchery management.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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