ORIGINAL ARTICLE

ISOLATION AND CHARACTERIZATION OF A NOVEL AEROMONAS PHAGE Φ265 AGAINST Β-LACTAMASE PRODUCING A. VERONII FOR APPLICATION AS A POTENTIAL BIOCONTROL AGENT

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ABSTRACT

Context: There is a long-term cognizance of emerging multidrug-resistant strains of *Aeromonas spp*. which is a matter of great concern for healthcare as well as environment. Therefore, there is a dire need to look for other natural alternative antibacterial management approaches. Hence, widespread lytic bacteriophages become considerably important.
Objective: This study reports isolation and partial characterization of a novel lytic bacteriophage from pond water against waterborne virulent pathogen *Aeromonas veronii*.
Methods: Enrichment technique using pond water along with bacterial culture was employed for the isolation of phage followed by double layered agar plaque assay for titre enumeration during characterization. The phage was characterized for stability to varied temperatures, pH and chloroform. The growth characteristics were also assessed by One-step growth curve.
Results: *Aeromonas* phage φ265 was isolated against virulent *A. veronii* which showed a lytic and broad biological spectrum activity against 12/13 *Aeromonas spp*. strains. One step growth curve analysis revealed that it has a latent period of approx. 30 min and an average burst size of ~ 40 PFU/infected cells. Further, temperature stability assay showed it to be well active at 4-55°C and at a pH range of 3-10. Phage lytic activity remained unaffected by the action of up to 20 % chloroform supplementation.
Conclusion- Bacteriophages concomitantly lie in abundance with their bacterial hosts. Such lytic phages can be fully characterized and harvested for their inherent antibacterial potential against pathogens in the same environment.

Keywords: Bacteriophage, lytic, plaque, antimicrobial resistance, virulence.

INTRODUCTION

Aeromonads are widespread, Gram-negative, rod-shaped, facultatively aerobic microbes commonly found in aquatic environments[1]. The taxonomy of genus *Aeromonas* is complex at the species level owing to its heterogeneity, but it belongs to the *Aeromonadaceae* family[11]. Their association with diseases is increasingly becoming popular as they are opportunistic pathogens, known to infect primarily fish, but also act as pathogens for humans and other aquatic animals. Given their prevalence in freshwater bodies, they now have become a challenging issue for cultured fish. Among the later, they can cause furunculosis, hemorrhagic septicemia or motile aeromonas septicemia, hemorrhagic enteritis, epizootic ulcerative syndrome, red sore disease, tail and fin rot, and lethargy[10] In humans, so far, intestinal and extraintestinal infections have been reported, ranging from mild sicknesses such as wound infections, gastroenteritis to severe conditions such as septicemia, respiratory infections, and necrotizing fasciitis[14] Direct contact or consumption of contaminated food or water or contaminated animal products are potential sources for their transmission to humans, causing severe infections. The clinical significance of aeromonads has been recorded in several invertebrate and vertebrate species including lizards, snakes, calves, bulls, dogs, frogs, and wild avian species[3]

Various studies have reported the prevalence of *Aeromonas veronii* among other aeromonads isolated from the natural water bodies and their associated antimicrobial resistance profiles[16,15,5]. Therefore, research interest has grown towards its more natural antibacterial agents, such as bacteriophages however, only few studies of bacteriophages against *A. veronii* have so far have been reported. Recently[2], reported the isolation of a T7-like phage belonging to family *Podoviridae* against *A. veronii* from pond water samples. In the current study, we describe the isolation and partial characterization of a novel phage *Aeromonas* phage φ265 targeted against concomitantly isolated *A. veronii* from pond water.

MATERIALS AND METHODS

Isolation of A. veronii and its antimicrobial sensitivity pattern

Bacterium *A. veronii* was isolated from fish culture pondwater around Hisar (Haryana, India) by surface inoculation of 100 µl of water sample over Starch-ampicillin agar (HiMedia) media and subsequent incubation at 35±1°C for 18-24 h. Yellow to honey-colored colonies of typically 3-5 mm diameter (suspected *Aeromonas* spp.) were also indicative of amylase positive reaction (presence of clear zone) after flooding with 5 ml of Lugol iodine solution[13]. These suspected *Aeromonas* colonies were identified phenotypically and biochemically and further confirmed on the basis of PCR amplified *gyr*B gene (~1100bp fragment) sequencing. Kirby-Bauer's procedure for antimicrobial resistance (AMR) assay using disc diffusion and double disc synergy methods was performed on Mueller-Hinton agar (HiMedia) media.

Isolation of lytic bacteriophage

Lytic phage specific to multidrug-resistant *Aeromonas veronii* was isolated from enrichment of same pondwater samples with overnight grown bacterial culture into 5X Nutrient broth (HiMedia) at 37°C for 18-24h. After incubation, the mixture was centrifuged at 10000 x g for 10 min. and supernatant was filtered through sterile 0.22 µm Millex® -GV syringe filter unit (Merck, Germany). Phage was isolated by spot test with 10µl of filtered sample followed by incubation at 37°C for 16-18 h. Putative *A. veronii* phage produced a clear zone in the spotted area.

Phage stock preparation and its titre enumeration

The lytic phage so visible was subjected to purification by selecting a single isolated plaque (~3mm diameter) and suspending it in 0.1 ml of SM buffer (50 mM Tris-Cl, pH 7.5, 99 mM NaCl, 8 mM MgSO4, 0.01% gelatin). Then, serial dilution technique was performed for the bacteriophage suspension followed by double agar layer plaque assay three consecutive times to obtain clonal selection. Further, titre estimation was done as described previously[2]. For phage stock preparation, freshly suspended phage was added to exponentially growing host culture and subsequent overnight incubation was given for 16-18 h. The bacterial lysate was the treated with chloroform, Dnase I and Rnase A in order to cause host inactivation and release of free phages in the suspension. Further, phage was precipitated using Polyethylene glycol and finally chloroform extraction was performed. Preservation of the isolated phage was carried out at -80°C as glycerol stock in National Centre for Veterinary Type Culture (NCVTC) with accession no. VTCCBPA 265.

Biological spectrum of phage Aeromonas phage φ265

Different *Aeromonas* spp. strains were subjected to spot test with the newly isolated bacteriophage Different tested species included various *A. veronii* isolates, following incubation, a clear lytic zone in the spotted area was considered as indicative of a clear lysis.

Sensitivity assay to chloroform, temperature and pH

Phage Aeromonas phage φ265 was incubated for 1 h at room temperature after treatment with chloroform dilution (5, 10, 20 and 30%) and the % inactivation was accounted as compared to the phage suspension kept in SM buffer held at 4°C. Phage stability assay at different temperatures (4, 25, 37, 45, 55, 60 and 80°C) was examined by incubation of the phage at each temperature for 1 h followed by titre estimation at the end of the incubation. Phage preparation held at 4°C acted as control. For stability evaluation of bacteriophage at various pH values, pH of the solution was adjusted with either 1 M HCl or 1 M NaOH to reach a desired pH range of 3–10. This phage suspension was then incubated at 37°C for 1 h. Phage suspension held at pH 7 acted as control. Double

One step growth curve

The one-step growth experiment was performed as described by Duarte et al., 2018. Briefly, a mid-log phase bacterial culture of *A. veronii* ($OD_{600\ nm}$=0.51) (9 ml) grown in Nutrient broth was incubated with 2 ml of phage BPA 265 at an MOI of 0.001 for adsorption without shaking at 37°C for 15 min. Thereafter, the mixture was centrifuged and pellet was resuspended in 10 ml sterile Nutrient broth at time zero (t=0). The tube contents were incubated at 37°C in a shaker incubator at 180 rpm for 2h. 0.1 ml aliquot was drawn at 10 min interval each and subjected to phage titration using double agar plaque assay after serial dilution in SM buffer. Plaques were counted after overnight incubation at 37°C.

RESULTS

Antimicrobial resistance pattern of A. veronii

The isolate was found to be resistant to four of the 15 tested antimicrobials according to a standard procedure based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) and found to be ESBL (Extended spectrum β-lactamases) and AmpC β-lactamases positive as determined by the double disc synergy methods.

Plaque morphology

A single plaque was isolated and purified against *A. veronii*. The plaque was having a 1mm lytic centre and the plaque morphology was uniform on all the nutrient agar plates with a marginal or no difference in the diameter (Fig I) and each plaque was characteristically surrounded by a turbid ring (bacterial growth) which was in turn surrounded by a lytic ring (Fig II). The average plaque diameter of phage was ~3mm.

Bacterial host range

The Aeromonas phage φ265 produced clear lytic zone on all of the different *A. veronii* isolates tested barring one as indicated in Table I.

Phage stability assays

Aeromonas phage φ265 was found to be relatively heat stable over a period of 1 h, between a temperature range of 4-55°C. However, at 60°C, a sharp decrease in phage activity was observed. When exposed to 80°C temperature, after 1 h a complete phage inactivation was observed (Fig III). Moreover, 37°C was found to be the optimum temperature for phage activity. Similarly, pH 7 was found to be the optimum and phage activity decreased while moving to both extremes, however, no complete inactivation was observed (Fig IV). After chloroform treatment, the infectivity of the phage remained similar to its original infectivity, indicating its resistance to chloroform for the tested range.

One step growth curve

As observed from the one step growth curve experiment, a triphasic growth curve depicting eclipse, latent and exponential growth period was observed. The latent period of the phage was ~30 minutes and average burst size was 40 PFU infected $cell^{-1}$ (FigV).

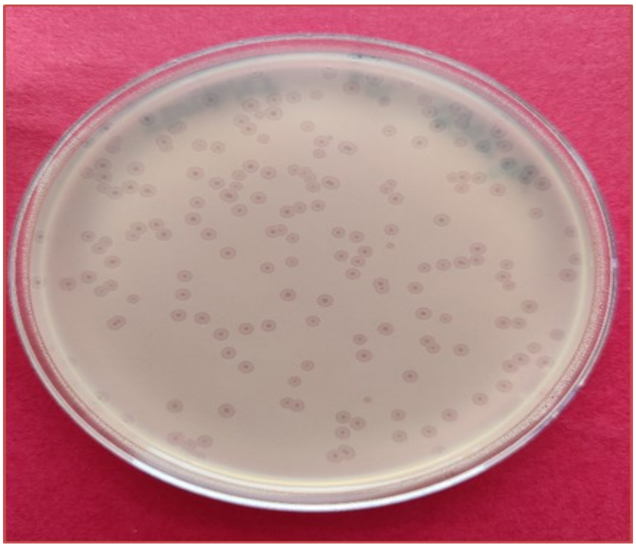

Fig I. Aeromonas phage φ265 plaques formed on double layer agar plates with the indicator host strain, *A. veronii* Aq 25

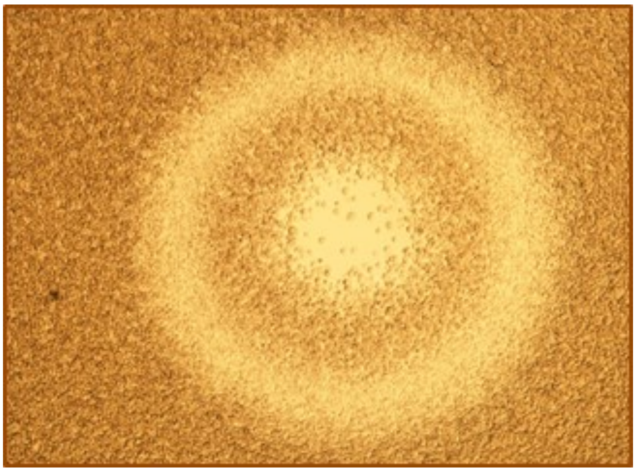

Fig II. Microscopic image of a single plaque morphology formed by Aeromonas phage φ265 (4X, NIKON; Eclipse H600L)

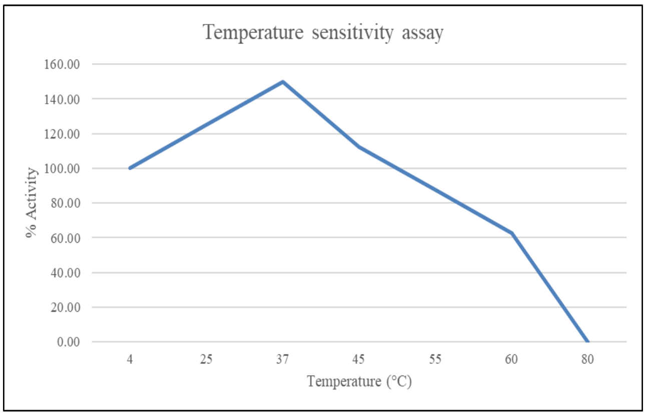

Fig III. Temperature stability assay for Aeromonas phage φ265

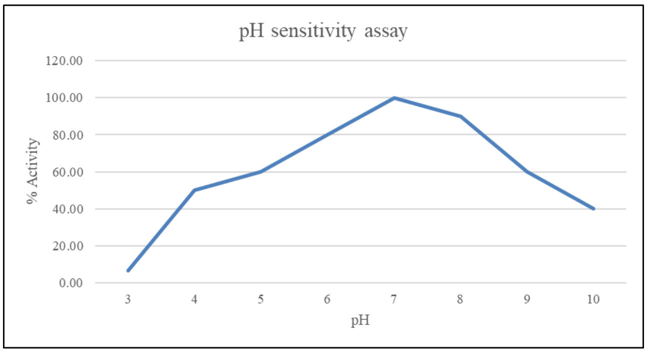

Fig IV. pH stability assay for Aeromonas phage φ265

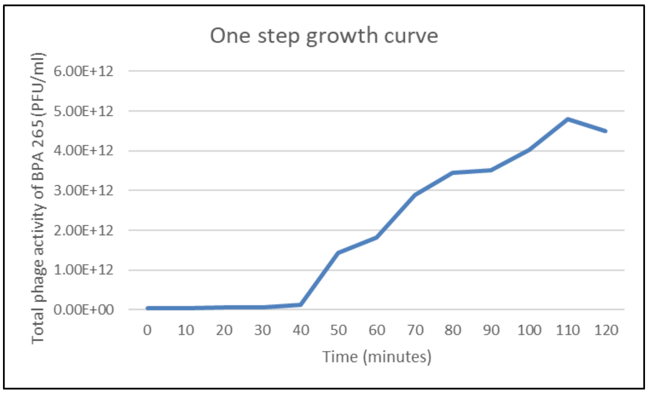

Fig V. One step growth experiment for Aeromonas phage φ265

DISCUSSION

In the current study, a pathogenic bacterium (*A. veronii*) was isolated from pond water system and identified biochemically as well as on the basis of amplified *gyr*B gene product sequence analysis. Its antimicrobial susceptibility assay showed that the bacterial isolate was ESBL and AmpC positive. Members of genus *Aeromonas* are known to be intrinsically resistant to β-lactam antibiotics such as ampicillin which can be attributed to the unstable nature of the β-lactam ring of such antibiotics[20]. Genus *Aeromonas* has been previously well reviewed[12]. A lytic bacteriophage φ265 producing a 3mm, circular plaque (however with a confluent bacterial ring inside) was isolated from the same pond water sample after enrichment against this virulent *A. veronii* strain. Bacteriophages specific to *Aeromonas spp.* are widespread in nature especially, freshwater environments and can be easily isolated and brought to action. Various studies have reported the efficacy of phage therapy to mitigate effects of *Aeromonas* spp. caused infections[18,6,8].

Table I: Determination of phage lytic spectrum spectrum by spot analysis on *A. veronii* strains

Bacterial species	Isolate ID	Host range of Aeromonas phage φ265
A. veronii	Aq 2C	-
A. veronii	Aq 8	+
A. veronii	Aq 16	+
A. veronii	Aq 25	+
A. veronii	Aq 31	+
A. veronii	Aq 38	+
A. veronii	Aq 47	+
A. veronii	Aq 50	+
A. veronii	Aq 51A	+
A. veronii	Aq 58	+
A. veronii bv. sobria	Aq 65	+
A. veronii	Aq 86	+
A. veronii	Aq 89	+

The novel bacteriophage - Aeromonas phage φ265 showed a latent period of ~30 min and an average burst size of 40 PFU (progeny particles) per infected cell. The bacteriophage was stable over a pH range of 3-10, being most active at pH 7 and a temperature range of 4-60°C, however further increase in temperature resulted in loss of activity. Results also showed that the phage was not sensitive to chloroform as it remained unaffected after treatment with up to 20% chloroform.

Bacterial host range was calculated to identify the phage activity spectrum against different *A. veronii* strains indicating that it is a wide host range phage. Use of broad host range phages is advantageous as most strains of the target bacteria are then likely to be covered under their biological spectrum[9] and it also reduces the chances of development of early bacterial resistance which otherwise becomes a limitation of the phage therapy[17,4]. Commercial scaling up of phage therapy can be undertaken as they are harmless, widespread in nature and highly specific towards their host[19]. Additionally, they can be used alone or synergistically in combination with antibiotics[7] which also gives successful implications.

CONCLUSION

The newly isolated Aeromonas phage φ265 reported in this study has many distinct characteristics such as short generation time, good burst size, wide host range, high stability over a wide range of pH, temperature and chloroform, which makes it an ideal and important biological control agent against the emerging drug-resistant and opportunistic pathogen *A. veronii*. Further genomic level characterization of phage is required to move further in exploring its antibacterial potential and their phage-host biology prior to its use in phage therapy.

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Conflicts of interest- The authors declare that there is no conflict of interest.

References

1. Abbott, S. L., Cheung, W. K., & Janda, J. M. The genus Aeromonas: biochemical characteristics, atypical reactions, and phenotypic identification schemes. *Journal of clinical microbiology*; 2003; *41*(6): 2348-2357.
2. Anand, T., Bera, B. C., Virmani, N., Vaid, R. K., Vashisth, M., & Tripathi, B. N. Isolation and characterization of a novel, T7-like phage against Aeromonas veronii. *Virus genes*; 2018; *54*(1): 160-164.
3. Bhowmick, U. D., & Bhattacharjee, S. Bacteriological, Clinical and Virulence Aspects of Aeromonas-associated Diseases in Humans. *Pol J Microbiol*; 2018; *67*(2): 137-149.
4. Castillo, D., Rørbo, N., Jørgensen, J., Lange, J., Tan, D., Kalatzis, P. G., & Middelboe, M. Phage defense mechanisms and their genomic and phenotypic implications in the fish pathogen Vibrio anguillarum. *FEMS microbiology ecology*; 2019; *95*(3): fiz004.
5. Dhanapala, P. M., Kalupahana, R. S., Kalupahana, A. W., Wijesekera, D. P. H., Kottawatta, S. A., Jayasekera, N. K., & Jagoda, S. D. S. Characterization and Antimicrobial Resistance of Environmental and Clinical Aeromonas Species Isolated from Fresh Water Ornamental Fish and Associated Farming Environment in Sri Lanka. *Microorganisms*; 2021; *9* (10): 2106.
6. Easwaran, M., Dananjaya, S. H. S., Park, S. C., Lee, J., Shin, H. J., & De Zoysa, M. Characterization of bacteriophage pAh -1 and its protective effects on experimental infection of Aeromonas hydrophila in Zebrafish (Danio rerio). *J Fish Dis*;2017; *40*(6): 841-846.
7. Gon Choudhury, T., Tharabenahalli Nagaraju, V., Gita, S., Paria, A., & Parhi, J. Advances in bacteriophage research for bacterial disease control in aquaculture. *Reviews in Fisheries Science & Aquaculture*; 2017; *25*(2): 113-125.
8. Gordola, K. M. C., Boctuanon, F. A. U., Diolata, R. A. A., Pedro, M. B. D., Gutierrez, T. A. D., Papa, R. D. S., & Papa, D. M. D. Evaluation of Phage Delivery Systems on Induced Motile Aeromonas Septicemia in Oreochromis niloticus. *PHAGE*; 2020; *1*(4): 189-197.
9. Hyman, P. Phages for phage therapy: isolation, characterization, and host range breadth. *Pharmaceuticals*; 2019; *12*(1): 35.
10. Janda, J. M., & Abbott, S. L. The genus Aeromonas: taxonomy, pathogenicity, and infection. *Clinical microbiology reviews*;2010; *23*(1): 35-73.
11. Navarro, A., & Martínez-Murcia, A. Phylogenetic analyses of the genus Aeromonas based on housekeeping gene sequencing and its influence on systematics. *Journal of applied microbiology*; 2018; *125*(3): 622-631.
12. Nokhwal, A., Ravikant, Anand. T. and Vaid, R. K. Aeromonas : an overview In L. T. Duncan (Ed.), *Advances in health and research,* Nova science publishers, Inc. New York, 2020, pp. 1-68.
13. Palumbo, S. A., Maxino, F., Williams, A. C., Buchanan, R. L., & Thayer, D. W. Starch-ampicillin agar for the quantitative detection of Aeromonas hydrophila. *Applied and Environmental Microbiology*;1985; *50*(4): 1027-1030.
14. Parker, J. L., & Shaw, J. G. Aeromonas spp. clinical microbiology and disease. *Journal of Infection*; 2011; *62*(2): 109-118.
15. Patil, H. J., Benet-Perelberg, A., Naor, A., Smirnov, M., Ofek, T., Nasser, A., & Cytryn, E. Evidence of increased antibiotic resistance in phylogenetically diverse Aeromonas isolates from semi-intensive fish ponds treated with antibiotics. *Frontiers in microbiology*; 2016; *7*: 1875.
16. Piotrowska, M., & Popowska, M. The prevalence of antibiotic resistance genes among Aeromonas species in aquatic environments. *Annals of microbiology*; 2014; *64*(3): 921-934.
17. Rao, B. M., & Lalitha, K. V. Bacteriophages for aquaculture: are they beneficial or inimical. *Aquaculture*; 2015; *437*: 146-154.
18. Silva, Y. J., Moreirinha, C., Pereira, C., Costa, L., Rocha, R. J., Cunha, Â., & Almeida, A. Biological control of Aeromonas salmonicida infection in juvenile Senegalese sole (Solea senegalensis) with Phage AS-A. *Aquaculture*; 2016; *2450*: 225-233.
19. Soliman, W. S., Shaapan, R. M., Mohamed, L. A., & Gayed, S. S. Recent biocontrol measures for fish bacterial diseases, in particular to probiotics, bio-encapsulated vaccines, and phage therapy. *Open veterinary journal*; 2019; *9*(3): 190-195.
20. Zdanowicz, M., Mudryk, Z. J., & Perliński, P. Abundance and antibiotic resistance of Aeromonas isolated from the water of three carp ponds. *Veterinary research communications;* 2020; *44*(1): 9-18.

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