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Short Communication

Isolation of a lytic bacteriophage against virulent *Aeromonas hydrophila* from an organized equine farm

Taruna Anand[1], Rajesh Kumar Vaid[1], Bidhan Ch. Bera[1], Jitender Singh[2], Sanjay Barua[1], Nitin Virmani[2], Rajukumar K.[3], Neeraj Kumar Yadav[1], Dinesh Nagar[1], Raj K. Singh[4] and B.N. Tripathi[2]

[1] Veterinary Type Culture Collection, Bagla Road, Hisar 125001, India
[2] ICAR – National Research Centre on Equines, Sirsa Road, Hisar, India
[3] ICAR – National Institute of High Security Animal Diseases, Bhopal, Madhya Pradesh, India
[4] ICAR – Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

A bacteriophage (VTCCBPA6) against a pathogenic strain of *Aeromonas hydrophila* was isolated from the sewage of an organized equine breeding farm. On the basis of TEM analysis, phage belonged to family Myoviridae. PCR amplification and sequence analysis of *gp*23 gene (encoding for major capsid protein) revealed phylogenetic resemblance to T4 like virus genus. Protein profiling by SDS-PAGE also indicated its resemblance to T4 like phage group. However, the comparison of its *gp*23 gene sequence with previously reported phages showed similarity with T4-like phages infecting Enterobacteriaceae instead of *Aeromonas* spp. Thus, to our knowledge, this report points toward the fact that a novel/evolved phage might exist in equine environment against *A. hydrophila*, which can be potentially used as a biocontrol agent.

Additional supporting information may be found in the online version of this article at the publisher's web-site

Abbreviations: PEG – polyethylene glycol; PCR – polymerase chain reaction

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Introduction

Out of 5100 phages examined by electron microscopy since 1959, about 96% are tailed phages [1]. Siphoviridae comprise majority, that is, 61% of these, but members of Myoviridae are most widespread and diverse as studied by TEM and metagenomics [2, 3]. T4 types are the best characterized out of family Myoviridae. It has been reported that out of ~200 best characterized T4 phages [4], 90% infect *Escherichia coli* or other enterobacteria and only 10% grow on phylogenetically more distant bacteria such as Aeromonas, Vibrio, Cyanobacteria, and so on [5]. T4 and T4-like phages have been mainly characterized by PCR amplification of *gp*23 gene encoding for major capsid protein [6–8]. However, there have been very few reports regarding isolation and characterization of T4 or T4-like phages infecting *Aeromonas hydrophila* [9–11] which is considered as a potential pathogen for aquatic and terrestrial environments [12, 13]. Additionally, its virulence potential has also been assessed in humans [14–16] and has been related with abortion in equines [17, 18]. In the present study, we isolated a pathogenic strain of *A. hydrophila* and corresponding bacteriophage from the sewage of an organized equine farm, which could serve as a biocontrol agent. The phage was further characterized at genetic level and phylogeny was established for T4 type relatedness.

Correspondence: Dr. Taruna Anand, Scientist (SS), Veterinary Type Culture Collection, ICAR-National Research Centre on Equines, Sirsa Road, Hisar-125001, India
E-mail: tanandbt@gmail.com
Phone: 01662-276748
Fax: 01662-276217

Materials and methods

Sample collection, bacterial isolation, and characterization

Sewage samples were collected from individual boxstalls of nine mares and combined drainage/sewage hole, from an organized equine breeding farm. Samples were diluted

and spread on NA and individual colonies with distinct morphology were picked and purified. Individual bacteria were used for bacteriophage enrichment using 5× nutrient broth followed by centrifugation (10,000*g*) and filtration through a 0.22-μm PVDF filter. Enriched filtrates were screened for phage activity using spot assay. The host bacteria showing phage activity was subjected to isolation of genomic DNA using ZR Fungal/Bacterial DNA Kit (Zymo Research Corporation, Irvine, CA, USA) and 16S rRNA was amplified [19], cloned into pGEM-T Easy vector, got sequenced commercially and submitted in GenBank database (accession no. KR067615). For identification of virulence factors, amplification of *aerolysin* gene and *lipase* gene of *A. hydrophila* were carried out as indicated previously [20, 21].

Bacteriophage enrichment, purification, and protein profiling

The bacteriophage was separated by picking a single plaque by double-agar layer technique [22] and purified in SM buffer (5.8 g/L of NaCl, 2.0 g/L of MgSO4, 50 ml/L of 1 M Tris, pH 7.5, 5 ml/L of presterilized 2% gelatin). Plaque characteristics were recorded and phage titre was determined. Concentrated phage was prepared by adding phage suspension (10^9 PFU) to exponentially growing bacteria followed by incubation under shaking conditions for 8–12 h at 37 °C. Host bacteria were inactivated by chloroform followed by Dnase I and Rnase (final concentration 1 μg/ml) treatment. Bacteriophage was concentrated using NaCl (1 M) and PEG 8000 (10% w/v) and purified by extraction with chloroform (1:1 v/v). The isolated bacteriophage preparation was submitted to VTCC vide Accession no. VTCCBPA6 (referred to as BPA6 henceforth). The protein profile of BPA6 was developed in 12% SDS-PAGE gel and analysed using AlphaEaseFC software (Alpha Innotech).

One-step growth curve and adsorption rate

A one-step growth experiment was performed to ascertain the burst size and latent period of BPA6 as reported earlier [23]. Phage adsorption rate determination was essentially the same as that used by Gallet et al. [24].

Molecular characterization and phylogenetic analysis

Gp23 gene (850 bp) was amplified from DNA extracted from BPA6, using Mzia1 and CAP8 primers [7], cloned in pGEM-T Easy vector and sequenced. Homology analysis was carried out using EMBOSS tool (http://www.ebi.ac.uk/services/dna-rna) and related sequences were analyzed using MEGA5 program [25]. Phylogenetic tree was constructed using Neighbor-Joining method with bootstrap analysis using 1000 replicates.

Electron microscopy

Electron micrography was performed by placing the phage suspension on formvar/carbon coated copper grids which were allowed to stand for 5 min. The grids were negatively stained with 1% phosphotungstic acid, pH 7 for 30 s and then excess stain was drained off using whatman filter paper. The grids were air dried and viewed on JEOL (JEM-1400, Japan) electron microscope operating at 80 Kv.

Results and discussion

In the present study, a total of 69 bacterial isolates were obtained from the boxstalls of nine mares and combined drainage/sewage hole. The bacteria were investigated through Gram's staining and colony morphology. Prominent bacteria obtained belonged to *Bacillus* spp., *E. coli*, and *Enterococcus* spp.; however, one isolate of *A. hydrophila* was also obtained from sewage of one boxstall. No phage activity was seen in any of the purified isolates barring *A. hydrophila*. Hence, *A. hydrophila* isolate was further characterized through biochemical analysis and 16s rRNA sequencing which confirmed it to be *Aeromonas hydrophila* spp. *dhakensis* (Accession no. VTCCBAA700). The isolate had virulence potential as confirmed by PCR amplification (Fig. 1A and B) of *aerolysin* gene (252 bp)—which encodes for an extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic properties and *lip* gene (760 bp)—which encodes a thermostable extracellular lipase.

A bacteriophage (BPA6) which formed plaques of 1–2 mm diameter was recovered after plating the enriched filtrate in the presence of host (VTCCBAA700). The plaques appeared translucent with clear centers. Upon visualization of phage concentrates by electron microscopy, phage morphology showed a complete bias toward family Myoviridae having an icosahedral head and a contractile tail with a base plate (Fig. 2) [4]. The dimensions of the phage were capsid dia: 62 nm; tail: 138 × 19 nm.

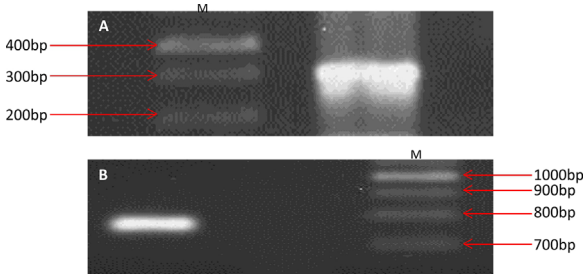

Figure 1. PCR detection of (A) aerolysin gene product (252 bp) and (B) lipase gene product (760 bp). (M) 100 bp DNA ladder.

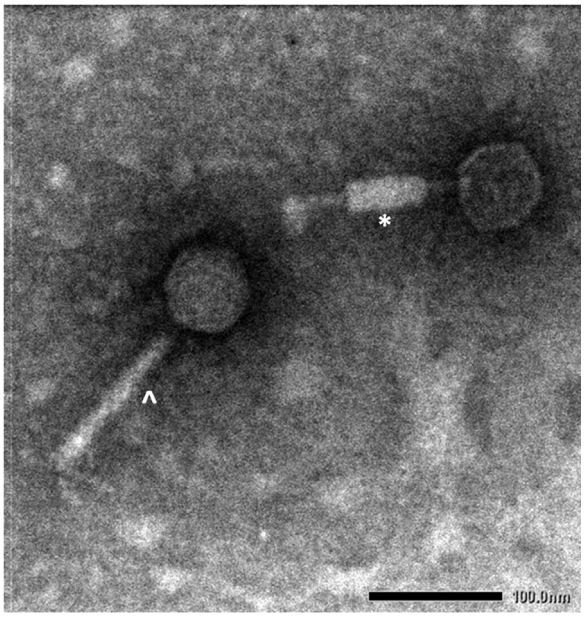

Figure 2. (^) Typical phage morphology of BPA6 observed under the electron microscope. (*) A phage particle with empty head and contracted tail.

For molecular characterization of the phage isolate, 850 bp central region of *gp23* gene was successfully amplified, cloned, and sequenced. The sequence data has been deposited in the GenBank database under accession no. KM111298. Homology analysis revealed 66 to ~98% similarity with closest Enterobacteriaceae phages, whereas identity was 32 to ~81% with *Aeromonas* phage isolates. Phylogenetic analysis (Fig. 3), revealed that there were four main clusters *viz*. G1 (T4-type), G2 (T-even), G3 (Pseudo T-evens), and G4 (Schizo T-evens) and our isolate belonged to T4-type phage cluster. Nine consensus aminoacid substitutions were observed in our isolate compared to closest Enterobacteriacae phage isolates (G1) except BP7, with which only four aminoacid changes were observed. The gp23 protein sequence of our isolate showed ~3.0% variation with phages of G1 cluster; however, within T-even group (G1 + G2), 35 aminoacid substitutions were observed altogether. It was also observed that G1 and G2 clusters consist of phages infecting bacteria of family Enterobacteriaceae and G3 and G4 clusters were mainly composed of phages against *Aeromonas* spp. Significant consensus sequence variations were observed between these four groups (Supporting Information Fig. S1).

The number of free phages in solution were decreased with time as observed by adsorption curve (Fig. 4A). The one step growth experiment of BPA6 lead to a triphasic curve (Fig. 4B) with a latent period of 10 min and the burst size was estimated to be 244 phage particles per cell. Structural protein profile of the isolated phage showed the existence of a protein ~48.5 kD (Fig. 5) which corresponds to major capsid protein gp23 of T4 type bacteriophages [26]. Another protein band of ~53 kD, probably coding for tail sheath protein was also observed. The host range of BPA6 was analyzed by checking the biological activity against *Aeromonas* spp. isolates (14 nos.) preserved in the repository and phage showed activity against 8/14 (57.1%) isolates by spot test.

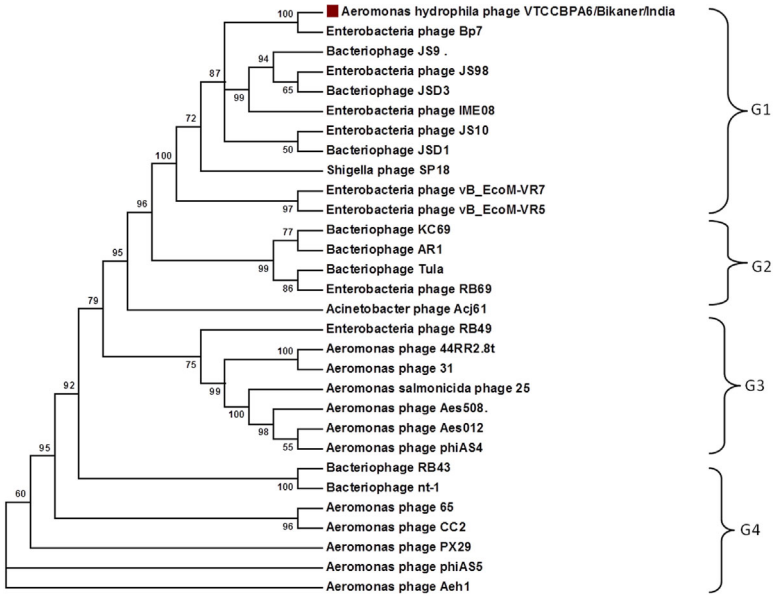

Figure 3. Phylogenetic analysis of *gp23* gene sequence of BPA6 against *Aeromonas hydrophila*.

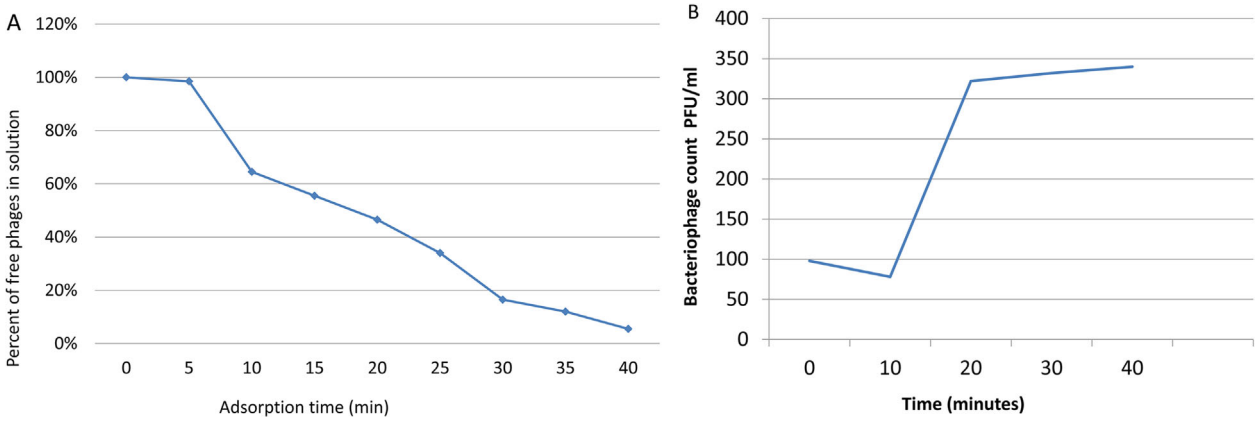

Figure 4. Adsorption curve (A) and one-step growth curve (B) of BPA6.

Aeromonas hydrophila has been reported for several pathological processes in equines such as septic arthritis, enteritis, and reproductive disorders including abortions in Thouroughbred mares [11, 21]. It has also been related to opportunistic infections in humans and is considered an important pathogen of aquatic animals [12, 13]. Many extracellular products of *A. hydrophila* such as aerolysin, enterotoxins, protease, and acetylcholinesterase have been considered as virulence factors in pathogenesis [27, 28]. The *aerolysin* and *lipase* gene products were detected in our *A. hydrophila* isolate, suggesting its pathogenic potential in the farm environment, which is in correlation with the previous study [18, 20, 21], where, however, the pathogenic indulgence of *A. hydrophila* was not determined at molecular level.

Although *A. hydrophila* has been studied at stretch, very little is known about phages infecting *Aeromonas* spp. Out of 20 phages (enlisted in NCBI) responsible for infecting Aeromonads, only *Aeromonas* phage Aeh1 and phage CC2 are known to infect *A. hydrophila*. Looking at the implications regarding persistence of such a microbe in equine environment [17, 18], we attempted to isolate phage against an equine farm isolate of *A. hydrophila* from the sewage of the same farm. A lytic phage was successfully recovered which belonged to family Myoviridae. The capsid measurements of BPA6 revealed that it is relatively smaller and uniform in dimensions as compared to other reported non-enterobacterial and enterobacterial phages [7, 8]. Furthermore, although the enterobacterial phage BP7 *gp23* major capsid gene sequence is phylogenetically closest to our isolate, it's capsid is significantly elongated ($93 \times 73 \times 106$ nm) [8]. Comparatively small capsid morphology of BPA6 (Fig. 2) fits the petite mutant criteria, however, we could not detect mutations in four locations (out of ten) as previously reported [29, 30]. Other nine consensus point mutations observed in our isolate could play some role in interactions with head accessory proteins which could result in variation in virion head structure [7]. However, the tail dimensions of Aeh1 [8], which is also an *A. hydrophila* phage, is almost equal in length with BPA6.

The phylogenetic relationship of BPA6 was assessed with available related phage isolates. The earlier report described the *gp23* sequence based classification of the T4-type phages

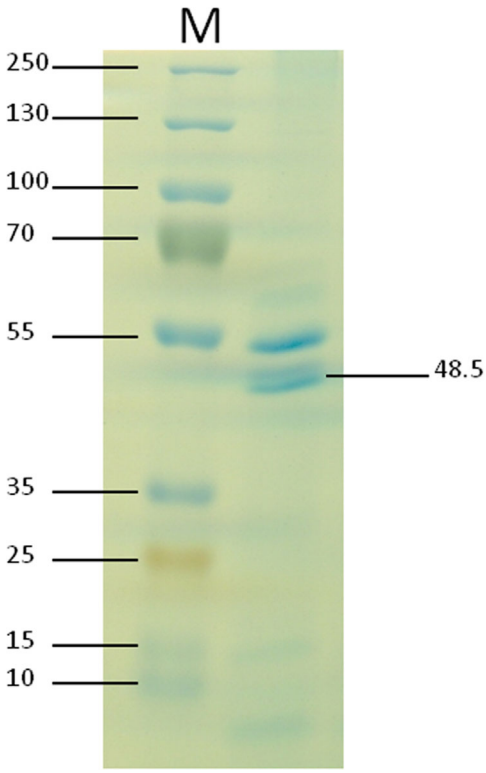

Figure 5. Protein profile of BPA6 in 12% SDS-PAGE. (M) Protein marker.

into three subgroups: T-even, pseudo T-evens, and schizo T-evens [7] and also suggested that the amino acid sequence of gp23 protein varies <10% within the T-even group. BPA6 also showed ~3% variation with T4 type phages. We observed that although the current BPA6 isolate was obtained from *A. hydrophila*, yet phylogenetically it was closest to *Enterobacteriaceae* phage BP7, instead of phages infecting Aeromonads. As 90% of the ~200 best characterized T4 phages [4] are reported to infect *E. coli* or other Enterobacteria and only 10% grow on phylogenetically more distant [5] bacteria including *Aeromonas* and given that the most phages can only infect a subset of bacterial species [31], we planned a small experiment to study the range of activity of isolated phage against 33 *E. coli* isolates, including equine isolates. However, no activity of the phage was found against them. Furthermore, the protein profile of the isolated phage also accorded the T4-like nature as demonstrated earlier [26]. These observations point towards either novelty of the BPA6 phage isolate or existence of mutations in certain T4-like Enterobacteria infecting phage which have somehow induced into it, the capability of infecting *A. hydrophila*. This aspect, however, needs to be further confirmed at the genomic level.

Concluding remarks

Here, we report isolation and partial characterization of a lytic bacteriophage against pathogenic *A. hydrophila*. The bacteriophage possesses novel characteristics and can be used as biocontrol agent in equine environment.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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