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Isolation of a novel cyanophage infecting potentially bloom-forming *Limnothrix* sp

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ABSTRACT

A filamentous cyanobacterium, identified as *Limnothrix* sp. VL01, and a novel lytic cyanophage Φ L-VL01 that infects it, were isolated from a shallow freshwater lake located in Goa, India. Cyanophage-infected *Limnothrix* sp. VL01 demonstrated reduced growth and increased fragmentation of filaments, compared to uninfected control. *Limnothrix* sp. is known to form blooms in freshwater lakes. Φ L-VL01 could be a significant agent in controlling such blooms.

Cyanobacterial blooms are on the increase worldwide, due to eutrophication of water bodies and rising global temperatures (Glibert, 2020). Filamentous cyanobacteria are frequently responsible for blooms, and lytic cyanophages are important agents of bloom control (Gerphagnon et al., 2015). *Limnothrix* sp. is a common bloom-forming filamentous cyanobacterium, with a worldwide distribution (Humpage et al., 2012; Yang et al., 2020). It can dominate the cyanobacterial community in shallow, eutrophic lakes, often along with other filamentous forms such as *Planktothrix agardhii*. Further, *Limnothrix* sp. has been identified as a water contaminant, with strains proposed to show toxicity (Daniels et al., 2014; Rose et al., 2018). The present study reports the isolation and characterization of *Limnothrix* sp. VL01 and its corresponding lytic cyanophage 'L-VL01', from a freshwater lake located in Goa, India.

Water samples were collected from Verna Lake (15.347689, 3.945478) situated in the state of Goa, India. Verna Lake is the source of the river Sal, the only river which originates in Goa, and is a site favoured by migratory birds. With an average depth of 4 m, the lake lies proximate to human habitation, is utilized in local agriculture and fishery activities, and is on the verge of eutrophication. From lake water samples, cyanobacterial cultures were isolated in BG-11 medium (Rippka et al., 1979), purified by streaking on BG-11 + 1% agar plates, and made axenic by treatment with Tienam (Sarchizian and Ardelean, 2010). Cultures were maintained at 25 °C, under a 16:8 light:dark cycle at irradiance between 10 and 20 μmol m^{-2} s^{-1}. The strain used in the present study was morphologically examined under a compound microscope. Molecular-level identification was carried out by amplification of the 16 S rDNA gene fragment (Nubel et al., 1997), followed by nucleotide BLAST analysis and generation of a Neighbour-Joining phylogenetic tree.

Water samples collected from Verna Lake were further screened for the presence of phages, using the isolated cyanobacteria as host. 0.22 μm-filtered water samples were added to host culture in the standard agar overlay technique (Bubeck and Pfitzner, 2005). Cyanophage plaques obtained on the agar plates were subjected to several rounds of purification, and thereafter, the pure phage stock was propagated in liquid and solid medium. Lysis of the host cyanobacteria in liquid medium was monitored by measuring the chlorophyll *a* concentration in test and control flasks over a period of 16 days. Chlorophyll *a* was extracted with methanol, measured spectrophotometrically and its concentration calculated by the following formula (Zavřel et al., 2015):

$$Chl_a\ [\mu g/ml] = 12{\cdot}9447\ (A_{665} - A_{720})$$

The phage lysate was concentrated with 10% w/v PEG 8000 and purified by cesium chloride density gradient ultracentrifugation. 10 μl of purified phage was adsorbed onto a formvar-coated copper grid and stained with 2% (w/v) uranyl acetate, followed by viewing with a Jeol JEM 2100 200 kV transmission electron microscope (Ackermann, 2009). Genomic DNA was extracted from the purified phage lysate, by the standard phenol-chloroform method (Sambrook and Russell, 2006). PCR-amplification of the *g20* gene, which encodes the portal protein of viruses belonging to the family *Myoviridae* was carried out with the

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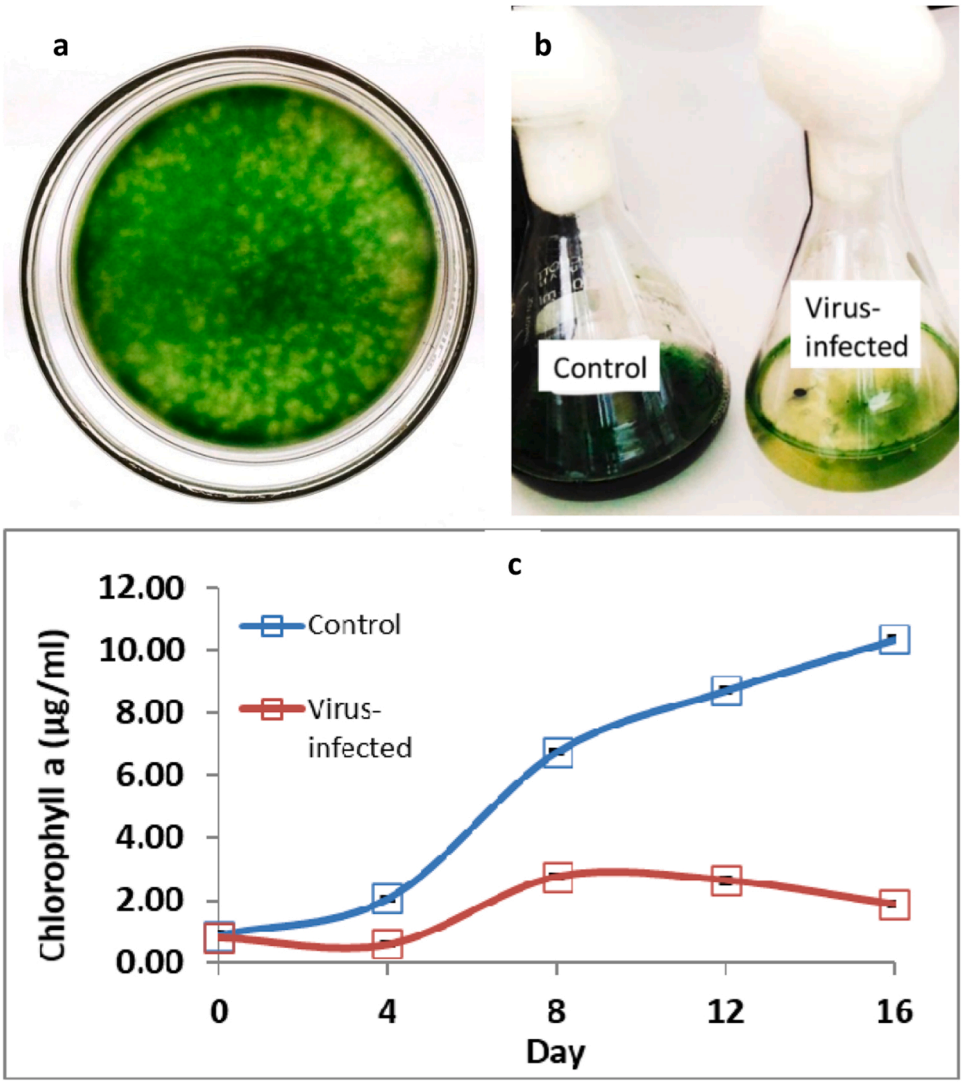

Fig. 1. Lysis of *Limnothrix* sp. VL01 by Φ L-VL01 in solid (**a**) and liquid (**b**) medium (**c**) Growth of control versus virus-infected *Limnothrix* sp. VL01, over 16 days, measured as chlorophyll *a* concentration.

primers CPS1 and CPS 8 (Wang et al., 2010).

Cyanophage-infected and uninfected cyanobacterial filaments were observed under 100X and 1000X magnification on a Lynx optical microscope. Additionally, the filaments collected on 0.2-µm polycarbonate filters, were dehydrated in an ethanol series, mounted on an aluminium stub, sputter-coated with gold particles and observed under a Zeiss Evo 18 scanning electron microscope.

The filamentous cyanobacterium obtained in axenic culture was designated strain VL01. Strain VL01 comprised non-heterocystous, solitary, unsheathed filaments consisting of narrow, long, cylindrical, cells with gas vacuoles (Fig. S1), These morphological features were in concurrence with the description of the genus *Limnothrix (*Meffert, 1988*)*. Nucleotide BLAST analysis of the 16 S rDNA sequence of strain VL01 (Accession number MN808643.1) demonstrated 99.80% identity with *Limnothrix* sp. Phylogenetic analysis with comparative homologous sequences from GenBank, using the Neighbor-Joining method, indicated that strain VL01 clustered with *Limnothrix redekei* (Fig. S2). Thus, the strain VL01 was designated *Limnothrix* sp. VL01.

The genus *Limnothrix* is primarily a freshwater form, which has been isolated from rivers, lakes and ponds in various locations of the world (Cunha de Oliveira et al., 2019; Zhu et al., 2012; Ojit et al., 2015). The *Limnothrix* strain used in the present study was isolated from lake water. The serial dilution method used for establishing a unialgal culture, inherently selects for the fastest-growing or most robust form within the specific aquatic niche. This indicates *Limnothrix sp.* VL01 to be a potential bloom-former. Total phosphorus concentrations of 0.15 mg/l have been observed in Verna Lake, which is six-fold above the threshold of 0.024 mg/l, above which increased proliferation of cyanobacterial blooms has been shown to be likely (Oram, 2019; Vuorio et al., 2020).

Subsequent screening of lake water samples against *Limnothrix sp.* VL01 by the agar overlay method, resulted in clear plaques, indicating the presence of a lytic phage (Fig. 1a). The plaques were purified and the isolated phage was designated Φ L-VL01. Φ L-VL01 demonstrated lysis of host in liquid medium (Fig. 1b), with an infective pattern similar to phages of other filamentous cyanobacteria (Gao E et al., 2009; Pollard and Young, 2010; Šulčius et al., 2015). Phage-infected host filaments turned from dark green to yellow, indicating their degradation. Further, phage-induced lysis of *Limnothrix* sp. VL01 was incomplete (Fig. 1b), and a residual growth of host filaments was observed. Incomplete lysis of filamentous host has been reported previously, in phage-infection of *Planktothrix agardhii* (Gao E et al., 2009) and *Aphanizomenon flos-aquae* (Šulčius et al., 2015). In comparison to the uninfected culture of *Limnothrix* sp. VL01, phage-infected culture demonstrated an 82% reduction in growth over 16 days, as indicated by chlorophyll *a* concentration (Fig. 1c). The difference in growth rate between control and infected culture was visible from the second day post-infection. The lysis time

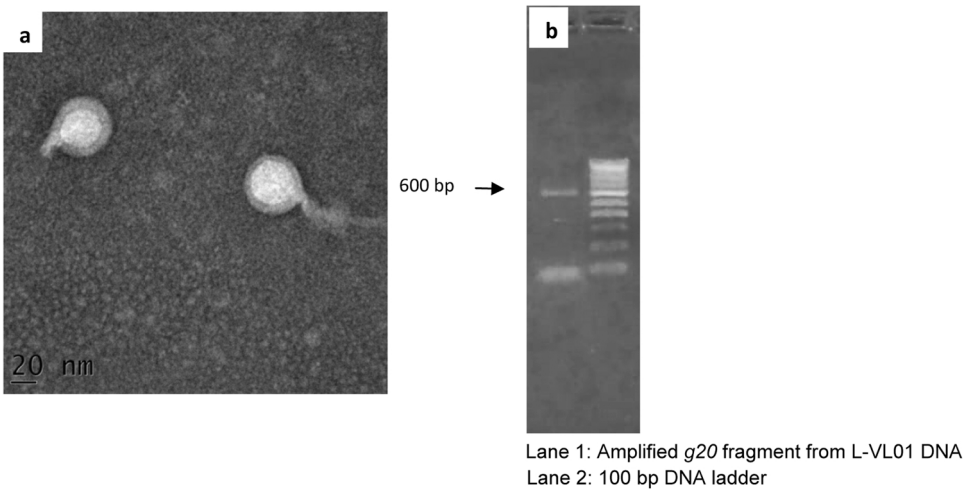

Fig. 2. (a) Transmission electron micrograph of Φ L-VL01(**b**) Amplification of *g20* gene fragment from genomic DNA of Φ L-VL01.

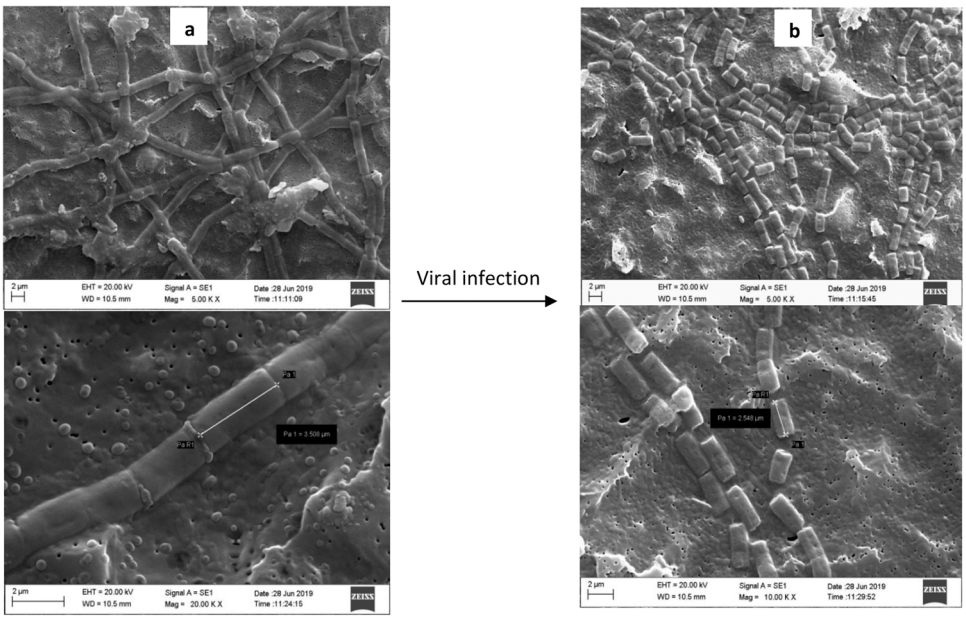

Fig. 3. Uninfected (a) and virus-infected (**b**) filaments of *Limnothrix* sp. VL01 under a scanning electron microscope.

reported for phages of filamentous cyanobacteria varies from twenty four hours (Zhou et al., 2013) to eight days (Gao E et al., 2009).

TEM analysis of Φ L-VL01 revealed phage particles having a head-and-tail structure. The icosahedral head measured approximately 50 nm in diameter (Fig. 2a**).** Cyanophages of filamentous cyanobacteria reported in literature have depicted a variety of head and tail morphologies (Table S1). The *g20* gene coding for the portal protein of myoviruses was amplified from the lysate of cyanophage L-VL01. Fig. 2b shows the PCR product of expected size around 600 bp. Presence of the *g20* marker indicates that L-VL01 belongs to the family *Myoviridae*.

Under a scanning electron microscope, control filaments of strain VL01 were intact, with cells joined end-to-end (Fig. 3a), whereas in the case of virus-infected filaments, cells were detached (Fig. 3b). A similar pattern had been observed under an optical microscope as well (Fig. S3). Fragmentation of host filaments following phage infection has been reported in several cyanobacterial species, notably, *Planktothrix* sp. Gao E et al. (2009), *Cylindrospermopsis raciborskii (*Steenhauer et al., 2016*)* and *Aphanizomenon flos-aquae (*Šulčius et al., 2015*)*. Fragmentation of cyanobacterial filaments (trichomes) into shorter "hormogonia" is a common mode of reproduction in filamentous cyanobacteria (Komárek and Johansen, 2015). In addition, the formation of hormogonia facilitates dispersal. Hence, the phenomenon of fragmentation following viral infection, could be a survival strategy of the host cyanobacterium.

The incidence of cyanobacterial blooms worldwide has increased, due to increased eutrophication and global warming. Although *Limnothrix* sp. is a known bloom former and toxin producer, there have been limited attempts to isolate phages infecting members of this genus. The only such report was of Φ L-DHS1, infecting *Limnothrix planktonica* strain DH5, which was isolated from Lake Donghu, China (Xiangling et al., 2015). In the context of the present study, the occurrence of *Limnothrix* blooms would be deleterious to the existing aquatic community including fish that thrive in Verna Lake. From this perspective, the isolation of a lytic cyanophage which could regulate uncontrolled proliferation of *Limnothrix* sp., assumes significance. The first reported *Limnothrix* phage, Φ L-DHS1, demonstrated structural uniqueness among tailed phages (Xiangling et al., 2015). The present study has highlighted certain functional aspects of the isolated phage, Φ L-VL01, such as its infectivity characteristics and potential to suppress the growth of its bloom-forming host. Attempts to increase the titre of the phage are in progress, which would facilitate its genome analysis, thus

giving further insight into the characteristics of Φ L-VL01.

Although freshwater systems were critical in the discovery of cyanophages (Safferman and Morris, 1963), a disproportionate number of cyanophage studies today have come from marine environments (Šulčius et al., 2019). The study of freshwater cyanophages from a functional perspective, is important, as they are distinct from their marine counterparts, even though they share the basic viral morphologies (Morimoto et al., 2020). In the Indian context, cyanobacterial blooms, though common, have not been extensively researched. Previous studies have largely concentrated on the production of microcystins (Chaturvedi et al., 2015; Sangolkar et al., 2009).

The isolation and characterization of novel viruses is a critical area in aquatic virus research. Laboratory studies on individual virus-host systems enable a better understanding of the contribution of viruses to the aquatic ecology (Weitz et al., 2013). At present, in the field of aquatic virology, the primary focus is on metagenomic investigations at the ecosystem level. Metagenomic studies are constantly uncovering an array of novel viruses. However, one crucial question that is not answered by metagenomic discovery is "Who is the host"? (Edwards et al., 2016) The basic information required about a new virus is which host it infects. This question is answered by traditional culture-based methods. Thus, laboratory isolation of viruses and their hosts would remain relevant, and continue to complement metagenomic studies.

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Ethics approval

Not applicable.

CRediT author contribution statement

Judith M. Noronha: Conceptualization, Investigation, Writing – original draft, Writing – review and editing. **Manguesh U. Gauns**: Writing – review and editing. **Sanjeev C. Ghadi**: Conceptualization, Writing – review and editing.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Code availability

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.limno.2022.125961.

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