Virus Research 263 (2019) 184–188

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Development and evaluation of taxon-specific primers for the selected *Caudovirales* taxa

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ARTICLE INFO

Keywords:
Bacteriophage
Taxonomy
PCR
TEM
DNA markers
Primers

ABSTRACT

The phage taxonomy is primarily based on the morphology derived from Transmission Electron Microscopic (TEM) studies. TEM based characterization is authentic and accepted by scientific community. However, TEM based identification is expensive and time consuming. After the phage isolation, before analysis TEM, a DNA based rapid method could be introduced. The DNA based method could dramatically reduce the number of samples analyzed by TEM and thereby increase the speed and reduce the cost of identification. In the present work, four environmental phage isolates were identified based on TEM studies and genome size. The identification of these four phages was validated using DNA based method. The taxon-specific DNA markers were identified through multiple sequence alignments. The primers were designed at conserved genes (DNA polymerase or integrase) of 4 different phage taxa *viz.* family *Ackermannviridae*, genus *Jerseyvirus*, genus *T4virus*, and genus *P22virus*. These primers were evaluated using both *in vitro* and *in silico* approach for the amplification of the target taxons. Majority of the primer sets were found to amplify member species of the targeted taxa *in vitro*. In *In silico* analysis, six primer sets intended for identification of family *Ackermannviridae* showed positive amplification of ≥86.7% classified species. Further, the primers targeting the genus *Jerseyvirus* and *T4virus* showed the amplification of 53.8% and ≥84.6% species, respectively. The present work is a case study performed to explore the possibility of use of taxon-specific primers for identification and taxonomic studies of newly isolated phages to supplement the TEM.

In the era of antimicrobial resistance, the researchers are in continuous search of the phages which can be used as the alternatives to the antibiotics. However, before such application, the collection of precise physiological and taxonomic information of the phages is crucial, as there are safety concerns linked with the use of lysogenic phages (de Melo et al., 2018).

The Transmission Electron Microscopy (TEM) is most notably used technique for bacteriophage (phage) taxonomy. This technique has been used conservatively for morphological characterization and the taxonomy of the phages since its invention (Ackermann, 2012, 2007; Ackermann and Prangishvili, 2012; Aziz et al., 2018). TEM is termed as "catch-all methods" in virus taxonomy due to its broad application and availability of the results for quick assessment (Vale et al., 2010). However, TEM is expensive and time-consuming as the researcher has to prepare the phage sample trickily and examine several sample areas to get the quality images (Eskelinen, 2008; Williams and Carter, 1996). In certain instances, the solitary reliance on TEM observations has led to anomalous phage taxonomy (Nelson, 2004). Till today there are no alternative methods to perform the taxonomic studies on phages besides TEM (Ackermann, 2013).

The number of phage genomes has been increased considerably due to current developments in Next-Generation Sequencing (NGS) technologies. With the leverage of ever-increasing phage genome data available at biological databases, the signature genes can be identified. Further, the signature genes may be amplified, sequenced and analyzed for the establishment of phage taxon (Clokie, 2009; Vallota-Eastman, 2017). This approach is reliable and progressively getting established. PCR and its variants of have been used in clinical diagnosis, diversity studies and detection of the viruses. The successful attempts have been made for the identification of phages by using the (Polymerase Chain

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https://doi.org/10.1016/j.virusres.2019.02.005
Received 4 September 2018; Received in revised form 16 January 2019; Accepted 11 February 2019
Available online 12 February 2019
0168-1702/

Reaction) PCR targeting several phage genes. DNA polymerase (gp43) and major capsid protein (gp23 and gp20) genes have been targeted to score the diversity of the marine viruses and cyanophages (Clokie, 2009). Primers based on the genes encoding for major capsid protein, tail tube protein, base plate wedge subunit and DNA polymerase have been previously used for the phage identification (Anand et al., 2018; Augustine et al., 2013; Clokie, 2009; Jäckel et al., 2017). Integrase has been used as a signature gene for detection of prophage-encoded toxin genes in bacteria and for the diversity assessment of temperate phages or prophages (Adriaenssens and Cowan, 2014; Balding et al., 2005; Colavecchio et al., 2017; Casas et al., 2006; Dwivedi et al., 2012; Yang et al., 2017). The amplification of phage DNA using PCR and the sequencing of the amplification products is expedient and may be used in complementation with TEM.

Four environmental phage isolates namely *Salmonella phage vB_SalM_PM10* (PM10), *vB_SalS_PM8* (PM8), *Alpha-a* and *vB_SalP_PM43* (PM43) were assigned to the families *Ackermannviridae*, *Siphoviridae*, *Myoviridae* and *Podoviridae*, respectively, based on the TEM observations. However, considering the limitations of the TEM, an attempt was made to develop taxon-specific DNA markers for the establishment of selected phage taxa (genera) which can be used alongside TEM to support the taxonomy. Based on genome size, partial genome sequencing and DNA homology analysis carried out with help of DNA markers, the aforesaid phages were assigned to the genera *Cba120virus*, *Jerseyvirus*, *T4virus* and *P22virus*, respectively.

The above-mentioned phages were isolated from sewage water samples and their purity was ensured (Newase et al., 2018). Further, the phages were propagated (Carey-Smith et al., 2006) and concentrated (Bao et al., 2011) to obtain high titer stocks. The phage particles from the stock (10^9–10^{10} PFU ml^{-1}) were washed twice with 0.1 M ammonium acetate (pH 7.0) by centrifugation at 25,000 × g for 75 min. The final suspension was mixed with 2% phosphotungstate (pH 7.2) and placed on carbon-coated copper grid (Ted-Pella Inc. California, USA). The grids were observed under LIBRA™ 120 TEM (Carl Zeiss, Oberkochen, Germany) operating at 120 kV. The head and tail measurements of the phage particles were taken using iTEM 5.1 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Phage genome size was determined by Pulse-field gel electrophoresis (Lingohr et al., 2009). The overall scheme for the minimum phage characterization required for taxon specific DNA marker development is shown in Fig. S1a.

The phage stock was treated with DNase I and RNase A added to the final concentration of 20 U ml^{-1} and 80 µg ml^{-1}, respectively. Using this treated phage stock DNA isolation was performed with phage DNA isolation kit (Norgen Biotech Corp., Canada) and/or by boiling in the water bath for 10 min. The host *Salmonella enterica* serovar Typhimurium (STm2501) DNA was extracted by boiling overnight grown culture for 10 min.

The primers for the genera *Cba120virus*, *Jerseyvirus*, *T4virus* and *P22virus* were developed on the genes at conserved regions attained by multiple sequence alignment (Table 1, Fig. S1b). The PCRs were performed in BIO-RAD T100™ thermal cycler. The reaction volume of 25 µl contained 1X PCR Master Mix (Thermo Scientific, India), 0.5 µg of the target and 1 pmol of forward and reverse primer. The temperature gradient PCR was carried out to determine the optimum annealing temperature for individual primer set. The PCR program was as follows: initial template denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 49–56 °C for 30 s, and 72 °C for 1 min. The final extension was carried out at 72 °C for 10 min. The primers from the previously reported study were also tested (Breitbart et al., 2004). All primer sets were evaluated for the amplification of phage PM10, PM8, PM43, Alpha-a, and host STm2501 DNA. The amplification was detected by electrophoresing the PCR product in 1.5% agarose gel. The selected amplicons were purified, sequenced, and their nucleotide sequences were deposited to GenBank database.

The primers developed in present study were also evaluated *in silico* using Primer-BLAST (Ye et al., 2012) (Performed 15 September 2018) against RefSeq representative viral genomes (Brister et al., 2015) of order *Caudovirales* (NCBI:txid28883) with the default parameters. Moreover, the primers were tested at insilico.ehu.eus/PCR/ (Bikandi et al., 2004) for amplification of DNA targets of taxon members. Each primer set was tested by allowing 0, 1 and 2 mismatches. The mismatches were not allowed in 10 nucleotides at 3′ end of the primer.

The morphology of phages was observed using TEM with respect to head, tail and the fine structures. Based on the TEM image analysis (Fig. 1) and genome size (Table S1) the phage isolates were assigned to four different families and putative genera as follows: phage PM10, family *Ackermannviridae* genus *Cba120virus* (Adriaenssens et al., 2018); phage PM8, family *Siphoviridae* genus *Jerseyvirus* (Anany et al., 2015); phage Alpha-a, family *Myoviridae* genus *T4virus* (Krupovic et al., 2016) and phage PM43, family *Podoviridae*; genus *P22virus* (Maniloff and Ackermann, 1998) (Table S1, Fig. S1b).

The phage taxon-specific primers were designed by aligning the multiple genomes of the classified phages available at GenBank (NCBI) database (Clark et al., 2016). In the present study, maximum possible homologous gene sequences in respective phage genera were included for the alignment. Gene selection was carried out in such a way that, at least 50% of the available gene sequences for each phage genera were covered. The detailed DNA data mining led to few potential genes for four different phage genera belonging to order *Caudovirales*. In genus *Cba120virus* genes encoding–topoisomerase, DNA helicase, host lysis protein and DNA polymerase were found to suitable for primer design. In genera *Jerseyvirus* and T4virus– gene DNA polymerase and in genus *P22virus*– gene integrase were found to be appropriate for primer design. Total 21 different primer sets with the average melting temperature of 50 °C were for four phage taxa were designed. The primers were evaluated *in vitro* against the phages which were identified based on comparative TEM genome analysis. For the genus *Cba120virus*, 12 primer sets were tested, and five primers sets were found to be positive. Primers for the Jerseyviruses (2 primer sets), T4viruses (3 primer sets) and P22viruses (2 primer sets) were tested. Two *Jerseyvirus*, 3 *T4virus* and 1 *P22virus* primer(s) showed positive *in vitro* amplification. The designed primers exclusively showed the amplification of the targeted genera members without any false positive amplification of unintended taxon species (Fig. 2, Table 1). The *in vitro* amplification by PCR was further confirmed by sequencing of PCR amplicons (GenBank MH165326, MH165327, MH165328, MH213126, MH182102 and MH299853). However, due to lack of the large collection of phages, all the 21 primer sets were tested by *in silico* Primer-BLAST against *Caudovirales* genomes (Table 2, Table S2). While performing *in silico* studies the mismatch settings were kept stringent to avoid the non-specific amplification. Primers TopoiIILA, DNAhelB and DNAhelC showed the amplification ≥86.3% taxon member species at family (*Ackermannviridae*) level, whereas DNA polymerase-based primers PolADNAPolA, PolADNAPolB and PolADNAPolC showed positive results for ≥93.3% taxon member species at subfamily (*Cvivirinae*) level. The primers for the genus *Jerseyvirus* and *T4virus* showed the amplification of ≥53.8% and > 84.6–92.3% member species, respectively (Table S2). Some of the unintended phage targets were amplified but were showing the different product size than that of targeted phages. The primer pairs proposed for phages belonging to family *Ackermannviridae* namely TopoiIILB, TopoiIILB, HostlysisProA and HostlysisProB showed less taxonomic coverage (Table 2). The primer set int.p22.ST64 T showed the good taxonomic coverage (83%) on P22viruses in insilico.ehu.eus/PCR/ analysis (Table S3). However, primers based on integrase gene failed in amplifying the P22viruses in Primer-BLAST. This may be attributed to the differences in the parameter applied to *in silico* PCR analyses by Primer-BLAST and insilico.ehu.eus/PCR/. The primers with a higher percentage of the amplification of the target taxon could be used for the identification of newly isolated phages (Table 2). The *in silico* amplification of the DNA targets takes very short time and provides a priory competence and certainty of the tested

Table 1
Primers used in the present study and their *in vitro* evaluation.

Primer	Target gene product	Primer forward, reverse (5'–3')	PCR assay against the phage isolates				
			PM8	PM10	PM43	Alpha-a	STm2501
TopoiIILA	Topoisomerase	ACCCATATCGGTGGTCCTCA,TCTGTGGGTAGCGGGTGATA	–	+	–	–	–
TopoiIILB	Topoisomerase	GACCGCTTTGGCCATTTTGT,AATCTTCTTGGCGTCAGGGG	–	–	–	–	–
TopoiIILC	Topoisomerase	AGTATCCGTTCAGCCGTTGG,TGTCGTGACACGGAACCATT	–	–	–	–	–
DNAhelA	DNA helicase	AGTCCTTGGTGCGTTCTACG,GTATGCTGCTGAAAAGGCGG	–	+	–	–	–
DNAhelB	DNA helicase	TGATCGACACTCCCTGTTGC,CAGGTCGTTGCCCATTTTGG	–	+	–	–	–
DNAhelC	DNA helicase	GTTCGAACATAACGGCCACG,TCATGGCGCGTTACTACAGG	–	+	–	–	–
HostlysisProA	Host lysis protein	TTTCCCTGGGTAGTTCGGGA,GGCCAGAAGTCACTACCCAC	–	–	–	–	–
HostlysisProB	Host lysis protein	ATCGGGTGACTCGTGTTCTG,ACGCGGTTATAGCCTTTGCT	–	–	–	–	–
PolADNApolA	DNA polymerase	TTGGCGTGACGGAGAAATGA,ACGTCGCCTTTACGGTCTTT	–	+	–	–	–
PolADNApolB	DNA polymerase	AGGCGCATATGTCATGGAGG,GCGCCCATTCACTGGTTTTT	–	+	–	–	–
PolADNApolC	DNA polymerase	ATGGGCTGGTTGGTGTAAGG,GCCACAATTGCTTGACCAGG	–	+	–	–	–
PolADNApolD	DNA polymerase	AAGGTCTGGAAGCGCGTAAA,GAGACCAACCAACCACGTCT	–	–	–	–	–
JerDP1	DNA polymerase	ACATCAAAACGGCGGTGTGC,AGCATGGCTAAGGATAAACG	+	–	–	–	–
JerDP2	DNA polymerase	GACGTAATGTCGCTTTTTGC,TGTGCAAGTATGGGACGCCA	+	–	–	–	–
t4-1	DNA polymerase	TGGAAGACATCGGTCTCGAAGCT,ATAGATACGCCATCAATAGA	–	–	–	+	–
t4-2	DNA polymerase	AACATCATTGACGTAGAATC,AACTTAACATATCGTTCAACT	–	–	–	+	–
t4-3	DNA polymerase	ACTATGATTTGCGAAATGCT,CATATTTCGCTATATCGTTCG	–	–	–	+	–
int.HK620.Sf6	Integrase	TGGCCTGTACCTTTATGTCC,AGGCCATTCGTGCTCGTTCA	–	–	–	–	–
int.p22.ST64T	Integrase	GATTTAAGCAGTCTCTTGG,ACAGGTGATTTAGTTCCGTC	–	–	+	–	–
HECTOR[a]	DNA polymerase	GCAAGCAACTTTACTGTG,CGAGAGATACACCAACGAA	–	–	–	–	–
PARIS[a]	DNA polymerase	ATACTACACGCTACTCTGG,GAGTGGCAAGAGGAGTTAT	–	–	–	–	–
int.F1G2	STm prophage	AAAAATCGCAAGGTGGCCGG,CAGCATGCATAGGTATGTCG	–	–	–	–	+

[a] Adapted from Breitbart et al. (2004).

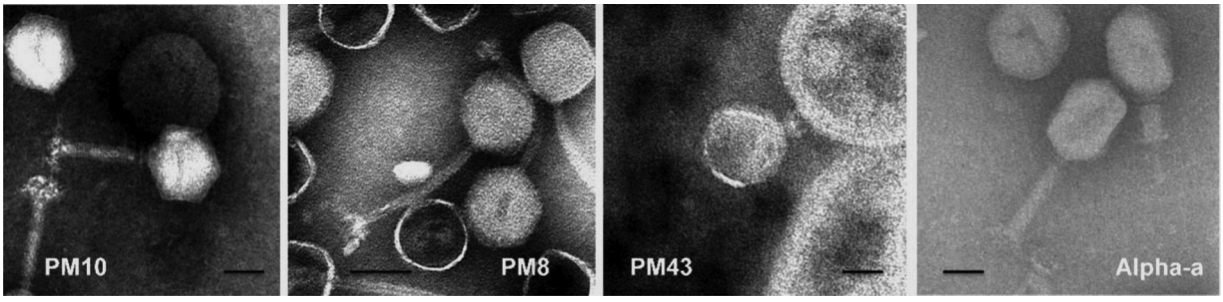

Fig. 1. TEM images of phage particles negatively stained with 2% phosphotungstate. Scale bar 50 nm.

primers for precise *in vitro* amplification. The *in silico* analysis result also suggests that for a given target taxon, the number of primers spanning the whole genome should be developed for assertive taxon identification.

Current phage taxonomy mainly relies on the morphological observations and is useful to classify phages into the families *viz. Ackermannviridae*, *Myoviridae*, *Siphoviridae* and *Podoviridae* based on capsid and tail type. The phages with the long contractile tail, long non-contractile tail and short tail are assigned to the families *Myoviridae*, *Siphoviridae* and *Podoviridae*, respectively. Recently, several phages from the *Myoviridae* family have been included in *Ackermannviridae* family showing several distinguishing morphological features described by Adriaenssens et al. (2012). Further, there are no guidelines on morphology based classification of the phages to hierarchies lower to the family level. However, genome size and GC content along with the genome- and protein-based homologies and phylogenies are being used for the classification of the phages to genus level. The genome region encoding for DNA polymerases from *Cba120virus*, *Jerseyvirus*, and *T4virus* was conserved amongst the all genus members. Additionally, DNA polymerase gene was found to be heterogeneous in genera *Cba120virus*, *Jerseyvirus*, and *T4virus*. Phage Orthologous Groups (POGs) based on the heterogeneity in the different taxon groups have

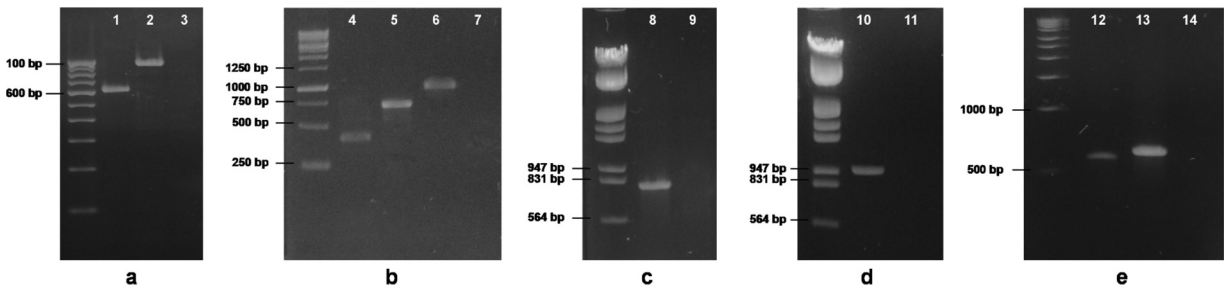

Fig. 2. Agarose gel (1.5%) of the PCR products obtained from **(a)** DNA helicase (lane 1), DNA polymerase (lane 2) from PM10 and negative control (lane 3); **(b)** DNA polymerase from PM8 (lane 4–6) and negative control (lane 7); **(c)** integrase from PM43 (lane 8) and negative control (lane 9); **(d)** integrase from STm2501 prophage (lane 10) and negative control (lane 11); **(e)** DNA polymerase from Alpha–a (lane 12 and 13) and negative control (lane 14).

Table 2
In silico amplification (Primer-BLAST) of the phages belonging to order *Caudovirales* using the primers developed in the present study.

Primer	Product length	Taxon	Taxon level	Classified species to the taxon level[a]	positive classified species[b]	Percent positive classified	Other taxon members amplified[c]
TopoiIILA	625	*Ackermannviridae*	Family	19	17	89.5	0
TopoiIILB	969	*Ackermannviridae*	Family	19	7	36.8	0
TopoiIILC	408	*Ackermannviridae*	Family	19	7	36.8	0
DNAhelA	416	*Cvivirinae*	Family	15	11	73.3	5
DNAhelB	427	*Cvivirinae*	Family	15	13	86.7	2
DNAhelC	633	*Ackermannviridae*	Family	19	18	94.7	1
HostlysisProA	564	*Vi1virus*	Genus	6	3	50.0	0
HostlysisProB	379	*Ackermannviridae*	Family	19	4	21.1	0
PolADNApolA	579	*Cvivirinae*	Subfamily	15	14	93.3	0
PolADNApolB	959	*Cvivirinae*	Subfamily	15	14	93.3	0
PolADNApolC	422	*Cvivirinae*	Subfamily	15	14	93.3	0
PolADNApolD	486	*Ackermannviridae*	Family	19	8	42.1	2
JerDP1	713	*Jerseyvirus*	Genus	13	7	53.8	1
JerDP2	405	*Jerseyvirus*	Genus	13	7	53.8	1
t4-1	583	*T4virus*	Genus	13	12	92.3	0
t4-1	583	*Moonvirus*	Genus	2	2	100.0	0
t4-2	596	*T4virus*	Genus	13	12	92.3	4
t4-3	645	*T4virus*	Genus	13	11	84.6	4
t4-3	645	*Rb69virus*	Genus	4	2	0.5	0
int.HK620.Sf6	952	*P22virus*	Genus	5	0	0.0	0
int.p22.ST64T	799	*P22virus*	Genus	5	0	0.0	0
HECTOR	533	*Podoviridae*	Family	124	0	0.0	5
PARIS	–	*Podoviridae*	Family	124	1	0.8	2

[a] ICTV (2017 release, https://talk.ictvonline.org/taxonomy/ accessed 28 August 2018).
[b] By Primer-BLAST analysis against viruses belonging to Caudovirales.
[c] Unintended taxons with the product size other than targeted taxon.

been suggested in a comprehensive bioinformatics study by Kristensen et al. (2013). Heterogeneity of DNA polymerase gene in phage genera *Cba120virus*, *Jerseyvirus*, and *T4virus* eliminated the possibility of the false positive identification. Therefore, the success taxon-specific primers primarily depend upon the morphological distinction and secondarily, upon selection of the DNA markers that are not shared by any other phage taxa under consideration. The selection of multiple sets of primers based on genome data would make phage taxonomy much easier and TEM could be used only to confirm the DNA based identification. In bacterial taxonomy 16S rRNA gene sequence is the primary tool for identification up to species level. However, the development and use of the universal phylogenetic marker for identification of phages is difficult due to high genetic diversity amongst bacterial viruses (Dwivedi et al., 2012). In phage taxonomy, as per the current practice, TEM and characteristics such as host range, topology and the genome size are used for the genus level classification of the phages. However, we found that the members of genera *Cba120virus*, *Jerseyvirus* and *T4virus* have additional morphological differences when compared to rest of the genera positioned under order *Caudovirales*. Therefore, before the taxon establishment and subsequent determination of taxon-specific marker genes, it is advisable to examine and compare the phage morphology with respect to additional features using available resources (Federhen, 2011; Hulo et al., 2010; Kropinski, 2009; Lefkowitz et al., 2017). The advancement of NGS and colossal genome database, it is easier than ever before to develop taxon-specific primers for the phages (Adriaenssens and Cowan, 2014). The use of taxon-specific DNA markers could revolutionize the phage taxonomy in an unprecedented way. Morphologically poorly-characterized phages and scarcity of their genome sequences would be major constraint in development of taxon-specific DNA markers. Additionally, phages show enormous DNA sequence diversity by virtue of the higher mutation rate than the bacteria by two orders of the magnitude (Drake et al., 1998).

In conclusion, the present study was an attempt to develop taxon-specific DNA markers. Four *Salmonella* phages from the environment were identified by TEM and taxon-specific DNA markers. Further, the DNA markers were validated by *in silico* PCR. This study may be of great use for rapid identification of newly isolated *Salmonella* or other enterobacteria phages. Our study is just a tip of iceberg in exploring the possibilities of using PCR for phage taxonomy. The dwindling price of DNA sequencing, robust data mining and genome sequence analysis tools would lead to a faster way of phage or virus identification. This DNA based identification may revolutionize viral taxonomy as 16S rRNA gene in bacterial taxonomy.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declarations of interest

The authors declare that they have no conflict of interest.

Acknowledgements

SN acknowledges the fellowship assistance given by DAE (GOI–E–156) under Bhabha Atomic Research Centre–Savitribai Phule Pune University Collaborative Research Ph. D. program. BPK is thankful to UGC, India for Emeritus Professor fellowship [No.F.6-6/2017 18/EMERITUS-2017-18-GEN-9819/(SA-II)]. The expert advice in the TEM analysis by Dr Anand Ballal, MBD, BARC, Mumbai is acknowledged. The authors are thankful to Mr Aniruddha Vaidya and Dr Uday Annapure, ICT, Matunga, Mumbai for providing phage Alpha-a stock and its TEM images.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.02.005.

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