

Morphological and Molecular Characterization of Indian Isolate of Brucella phage

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A phage showing lytic activity against strains of *Brucella abortus* was characterized for morphology, structural proteins and genome fingerprints. In electron microscopy, the phage showed presence of an icosahedral head and a short non contractile tail and was found indistinguishable from the family *Podoviridae*. Structural protein profiling of the phage by SDS-PAGE revealed nine proteins ranging from 16-114 kDa. The phage was found to possess double stranded DNA as genetic material. Genomic fingerprinting was carried out by Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD). Out of 19 restriction endonucleases used, the phage genome was sensitive to digestion with 15 enzymes yielding distinct and highly reproducible banding patterns. Molecular size of phage genome ranged from 56 to 81 kbp. The *in silico* comparison of RFLP patterns with reference phage *Tbilisi* and *Weybridge* revealed significant difference in the genomic sequence. The RAPD finger prints produced by 12 primers were distinct and highly reproducible. The generated finger prints can be use in high resolution brucellaphage diversity studies.

Key words: Brucellaphage, Restriction fragment length polymorphism,
Random amplified polymorphic DNA.

Brucellosis, an economically important re-emerging zoonotic infectious disease worldwide, is caused by Gram negative facultative intracellular bacterial organisms of the genus *Brucella* that are pathogenic for a wide variety of animals and human

beings (Mantur and Amarnath, 2008; Manish *et al.*, 2013). The disease is widespread in India and has been reported from almost all states (Renukaradhya *et al.*, 2002; Aulakh *et al.*, 2008). It has a considerable impact on human and animal health, as well as socioeconomic impacts, especially, in which rural income relies largely on livestock breeding and dairy products since the disease causes abortions, premature births, decreased milk production and repeat breeding leading to temporary or permanent infertility in infected livestock (Corbel 1997; Boral *et al.*, 2009; Lopes *et al.*, 2010; Verma 2013).

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The bacteriophages that infect *Brucella* species are called Brucellaphages. The existence of bacteriophage active against *brucella* was claimed since 1960s when phage Tbilisi (Tb) was discovered (Pickett and Nelson, 1951; Drozhevskina, 1956; Parnas *et al.*, 1958). Brucellaphages are linear non-enveloped double-stranded DNA viruses with a 50–80 nm diameter icosahedral head and a 10–15 × 7–9 nm long noncontractile tail. They are classified as members of C1 Group morphology belonging to the family *Podoviridae*, order *Caudovirales* (McDuff *et al.*, 1962; Ackermann, 2007).

Lytic bacteriophages infecting *Brucella* are routinely used in diagnostic applications to confirm the identity of *Brucella* species. They are host specific, hence a robust typing system was developed using seven reference brucellaphages (Corbel and Thomas, 1980). Phage groups prototypes are Tbilisi (Tb), Weybridge (Wb), Firenze (Fi), R/C, Izatnagar (Iz) and Berkeley (BK) and Nepean (Np) (Corbel, 1987; Rigby *et al.*, 1989; Hammerl *et al.*, 2014). These Brucellaphages differ mainly in the species of *Brucella* which they infect and their activity is highly sensitive to smooth-rough variation in their host brucellae. Tb and Fi phages replicate in smooth *B. abortus* (Rigby *et al.*, 1989), Tb phage lyses *B. abortus* at RTD (Routine Test Dilution), while R/C phage doesn't lyse *B. abortus* or *B. melitensis* at RTD. They reported phage lyses characters as a mean of differentiation among *Brucella* species. Wb replicates in smooth strains of *B. suis*, *B. abortus* and *B. neotomae* (Morris and Corbel, 1973), Iz replicates in smooth strains *B. melitensis*, *B. suis*, *B. abortus*, rough strains of *B. melitensis* and *B. suis* and, to a lesser extent *B. ovis* (Corbel *et al.*, 1988; Pandey *et al.*, 2013). These lytic activity patterns are important in diagnosis and therapy of the disease caused by a particular species.

In the recent years, molecular characterization of reference Tbilisi phage DNA (Zhu *et al.*, 2009), comparative genomic analysis of two brucellaphages Tb and Pr isolated at two distant places (Flores *et al.*, 2012) and comparative whole genome analysis of six diagnostic brucellaphages (Tb, Fi, Wb, Bk, R/C and S708) had been reported (Farlow *et al.*, 2014).

In the present study, the brucellaphage 'ΦLd' isolated in 2010 against *Brucella abortus* S

19 from sewage sample of a dairy farm in Ludhiana, India (Chachra *et al.*, 2012; Pandey *et al.*, 2013). The phage was examined for morphology using Electron microscopy and for molecular profiling by DNA restriction endonucleases, random amplified polymorphic DNA and SDS-PAGE to facilitate its wider use in various diagnostic and immunotherapeutic applications.

MATERIALS AND METHOD

Bacterial strains

B. abortus S19 are obtained from the Brucella Referral Laboratory, Division of Veterinary Public Health, IVRI, Izatnagar. The identity of each *Brucella* strain was confirmed by its morphological, cultural and biochemical and serological properties. The strains were maintained by periodic sub-culturing on Brucella agar (Difco) slopes.

Brucellaphage

A phage showing consistent lytic activity against *B. abortus* S19 was isolated in the Department of Veterinary Microbiology, Veterinary College, Guru Angad Dev Veterinary and Animal Science University, Ludhiana, Punjab, India and used for the present studies.

Revival, propagation and master stock

The stock suspension of the phage was sterilized by passing through 0.22 µm PVDF filter (Millipore) before confirming its lytic activity against S19 on Brucella medium soft agar overlay (Sambrook *et al.*, 1989). A plate showing plaques visible to the naked eyes was selected for further purification of the phage through three repeated cycles of streaking a single plaque on soft Brucella agar overlay inoculated with purity-checked, 48h incubated broth culture of S19. Using SM buffer (50 mM Tris-Cl [pH7.5], 0.1 M NaCl, 8 mM MgSO₄·7H₂O, 0.01% Gelatin) the purified phage was finally harvested from several plates showing clear lysis around streaked lines, and pooled. The harvested suspension pool (about 25 ml) was clarified by centrifugation at 4°C and filtered through 0.22 µm PVDF filters. The phage count of the pool was determined before storing it in small aliquots at 4°C as master stock. The phage count was determined by usual serial dilution method on NZCYM soft agar (0.7%) having fresh *Brucella* (S 19 strain) PBS suspension. The plaques formed on

agar plate's dilution wise were counted and the titre was expressed in plaque forming units (pfu)/ml.

Concentration of bacteriophage

Phage stock having titre $>10^9$ pfu/ml was concentrated according to the method of Yamamoto *et al.*, 1970 with some modifications. Briefly, NaCl and polyethylene glycol (PEG) 8000 were added to filtered phage stock to a final concentration of 1 M and 10% respectively and kept at 4°C overnight. The precipitate was centrifuged at 10,000 g for 10 min and pellet was resuspended in 0.01 original volume sterile SM diluent. This concentrated phage was stored at 4°C for future use in electron microscopy, nucleic acid isolation and protein profiling.

Electron microscopy

The concentrated phage was fixed by adding 1/10th volume of 2.5% buffered glutaraldehyde solution for minutes at room temperature. About 20 µl of fixed phage was poured on the surface of carbon coated copper grid and left for 2-5 min. For negative staining, 20 µl of 2% phosphotungstic acid (PTA) was to the grid surface. After 10-15 minutes, the stained grids were viewed under transmission electron microscope (Morgagni 268D, FEI Company). TEM was performed at All India Institute of Medical Sciences (AIIMS), New Delhi.

SDS-PAGE of proteins

The profiles of phage structural proteins were analysed by SDS-PAGE as described by Laemmli, 1970. The PEG concentrated phage was mixed with double volume of acetone and kept at -20°C overnight for precipitation of proteins. The precipitate was centrifuged at 10,000 g for 10 min and pellet was resuspended in 0.1 original volume of sterile water. Phage protein sample was mixed with equal volume of sample buffer (100mM Tris-HCl; pH 6.8, 4 % SDS, 20% glycerol, 200mM Beta-mercaptoethanol and 0.2% bromophenol blue) and denatured in a boiling water bath for 5 min. Proteins were electrophoresed in an SDS-polyacrylamide gel (12%) and protein bands were visualized after staining the gel with Coomassie blue. Protein molecular weight marker (Purgene cat # PG-PMT0782) was included in the run with samples to determine molecular weights/size of bands obtained.

Isolation and characterization of Nucleic acid

Concentrated phage lysate was treated with DNase I and RNase A at 37°C for 1 hour to rule out the bacterial DNA and RNA and then processed for phage nucleic acid extraction using Promega Wizard DNA cleanup kit following the manufacturer's protocol with minor modification. Quality and quantity of extracted nucleic acid was checked on 0.7% agarose gel electrophoresis at 5 V/cm by running along with lambda DNA undigested and lambda *EcoRI* + *HindIII* digested marker. The extracted nucleic acid was subjected to a digestion with DNase I, RNase A and S1 nuclease (Thermo-scientific) to confirm the type of nucleic acid. Each digestion was performed in 20µl of volume which consists of ~250 ng nucleic acid and 1 µl of DNase I/ RNase A/ S1 nuclease (5U/µl) and kept the reaction at 37°C for 30 min. Subsequently, the reactions were terminated by addition of EDTA (10 mmol/L final concentration) and digested products were separated on 0.7% agarose gel.

Genomic fingerprinting by Restriction Fragment Length Polymorphism (RFLP)

To develop the genetic fingerprints and assessment of genetic diversity of phage ΦLd from reference Brucellaphage Tb and Wb, and to determine the approximate genome sizes from the fragments generated, restriction digestion analyses were carried out with 19 restriction enzymes. The restriction enzymes BglI, SalI, SacI, Aval, ApaI, NotI, EcoRII, EcoRV, KpnI, NcoI, XbaI, Bsp119I (BstBI), HindIII, XhoI, PstI, EcoRI, BamHI, Eco31I (BsaI) and AluI were used according to manufacturer's (MBI Fermentas) instructions. After 3 hours of digestion, digested phage DNA fragments were separated on 0.7% (w/v) agarose gel containing 0.5 mg/ml ethidium bromide at a constant voltage of 3mV/cm in TAE buffer. The fragments of phage DNA digested by restriction endonucleases were calculated from relative mobility to standard size markers using image lab Software. Finally individual fragment sizes were added up for each enzyme to calculate the approximate phage genome size.

Genomic fingerprinting by RAPD analysis

RAPD was carried out as per protocol described by Williams *et al.*, 1990 using 18 random primers of series A, N and Q from Operon

Technologies (Alameda, USA). PCR with single primer was carried out in a final volume of 20 µl containing 2.0 µl of 10x assay buffer (Tris-Cl; pH 9.0, KCl and gelatin) 1.5 mM MgCl₂, 200 µM of each dNTPs (MBI Fermentas Inc. USA) 10 pM of primer, 1.0 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 30 ng of template DNA. The amplification reaction was performed in Thermal Cycler S1000 (Biorad, California US) the reaction conditions consisted of initial denaturation at 94°C for 4 min followed by 40 cycles each of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C. The final step consisted of one cycle of 7 min at 72°C for complete polymerization. After completion of the PCR, 2.5 µl of 6X loading dye (MBI Ferment Inc. USA) was added to the amplified products and were electrophoresed in a 1.5% (m/v) agarose (MBI Ferment Inc. USA) gels with 1x TAE buffer, stained with ethidium bromide and visualised under UV light. Gel photographs were scanned by using Molecular Imager Gel Doc XR+ (Biorad, Berkeley, California US). The sizes of the amplification products were estimated by comparing them to standard DNA ladder (Gene Ruler 100 bp plus DNA ladder Bangalore genei and 1 Kb DNA ladder; MBI Ferment Inc. USA).

Data analysis

The gel images of RFLP, RAPD and SDS-PAGE were analyzed using image Lab software (Biorad) and their size in terms of Base pair/ kilo base pair/ kDa were determined with reference to the standard ladder/ marker.

RESULTS AND DISCUSSION

Phage morphology

The brucellaphage ϕ Ld was examined by electron microscope in order to study its morphology. Based on TEM images, the phage has icosahedral capsid of 56 ± 2 nm in diameter and a short non-contractile tail of 18 ± 2 nm long and 7 ± 0.5 nm thick (Figure 1a and 1b). The size and shape resembles another Brucella phage Tbilisi and the other phages isolated elsewhere (Calderone and Pickett, 1965; McDuff *et al.*, 1962; Zhu *et al.*, 2009; Chachra *et al.*, 2012). According to International Committee on Taxonomy of viruses, these types of phages were assigned to family *Podoviridae*, order *Caudovirales* (Mathews, 1982; Ackermann, 1999). These phages

were further classified into subcategory morphotype C, subdivision C1 in the classification proposed by Bradley, 1967 and Ackermann, 2001.

Protein profiling of phage

The protein composition of brucellaphage ϕ Ld was characterized by SDS-PAGE, on which ten bands of structural proteins were obtained with a molecular weight of 114, 63, 56, 49, 39, 37, 32, 28 18 and 10 kDa (Figure 2). The most prominent bands, likely to represent the major capsid proteins, were 39 kDa, and 37 kDa in phage. Similarly, Zhu *et al* (2009) observed nine bands, ranging from 40 to 85 kDa of the structural proteins of Tbilisi phage and Flores *et al* (2012) observed about eight bands of structural proteins in Tb and Pr phage ranging from 27 to 88 kDa. While Pandey *et al* (2013) observed four bands with a molecular weight of 65.98, 60.46, 48.56 and 43.97 kDa from the isolated brucellaphage.

Nucleic acid characterization

The extracted nucleic acid from brucellaphage ϕ Ld showed as one distinct band upon electrophoresis on a 0.7% agarose gel. This indicated that the quality of extracted nucleic acid was pure and contained no DNA from the bacterial chromosome. For estimating the size of phage nucleic acid, it was loaded adjacent with the same quantity of control undigested phage lambda (λ) DNA and Lambda EcoRI/HindIII digested lambda DNA marker. The gel image taken after 6 and 9 hours of electrophoresis (Figure 3a and 3b) shows difference in migration pattern between brucellaphage nucleic acid and undigested lambda DNA, indicating that the brucellaphage nucleic acid was higher or equal in molecular size as compared to lambda undigested DNA (Mol. wt. about 48.5 Kbp).

For confirmation of type of nucleic acid, Phage nucleic acid when digested with DNaseI, RNaseA and S1 nuclease was found to be sensitive to DNase I, but was resistant to digestion by RNase A and S1 nuclease. It was concluded that phage contain double-stranded linear DNA as genomic material (Figure 3c).

Genome fingerprinting by RFLP analysis

For many organisms genetic maps are not available and relatively little is known of their molecular biology. In these circumstances molecular techniques like RFLPs and RAPD are most commonly used to decipher the genetic

Table 1. Genomic fingerprinting of brucellaphage ϕ Ld by RFLP and its comparison with reference phages *Tbilisi* and *Weybridge*

S. No	Name of enzyme	Enzyme digestion site	No. of fragments obtained from isolated phage	Details of size of each fragments in base pair generated by analysis using image lab software as compared to standard marker	Total sum of fragment size in base pair	No. of fragments obtained <i>in silico</i> by reference phage	Tb (genome size 41,148bp)	Wb (genome size 38253 bp)
1	BglII	GCCN	21	19079, 5053, 4445, 3263, 3000, 2740, 2371, 2166, 1964, 1800, 1516, 1409, 1239, 1107, 993, 864, 768, 739, 676, 613, 564	56369	18	18	16
2	Sall	NNN	10	19079, 9194, 7767, 7089, 6529, 5814, 4986, 4268, 1251, 739	66716	3	3	2
3	SacI	GAGCTC	10	17413, 8908, 7767, 5026, 4624, 4182, 2643, 2488, 2286, 1545	56882	4	4	4
4	AvaI	CYCGRG	16	17413, 10373, 8510, 6875, 6275, 5093, 4491, 3971, 3186, 2910, 2328, 2101, 1568, 1228, 912, 564	77798	3	3	2
5	Apal	GGGCCC	1 (no cut)	> 21226	NA	3	3	3
6	NotI	GCGGCCGC	2	e"21226, 18669	NA	1(No cut)	1(No cut)	1(No cut)
7	EcoRII	5'...“jC C ' W G C ...3	21	17413, 7534, 6275, 5080, 4774, 4006, 3344, 3205, 2910, 2533, 2357, 2192, 1988, 1862, 1602, 1553, 1423, 1281, 983, 885, 768	73968	46	46	46
8	EcoRV	GATATC	18	17413, 8255, 6772, 5904, 4986, 4006, 3129, 2458, 2287, 1656, 1584, 1402, 1293, 1182, 1060, 915, 772, 564	65638	12	12	11
9	KpnI	GGTACC	10	16068, 12311, 11367, 9760, 8007, 6568, 5972, 5268, 4090, 2043	81454	5	5	4
10	NcoI	CCATGG	18	19079, 13036, 6875, 6379, 3820, 3487, 2920, 2578, 2403, 1874, 1676, 1535, 1350, 1183, 981, 882, 778, 548	71384	8	8	8
11	XbaI	TCTAGA	4	e"21226, 13195, 10352, 1957	e"45504	2	2	2
12	Bsp119I (BstBI)	TTCGAA	5	e"21226, 18660, 8985, 6358, 3805, 2683	e"60491	9	9	8
13	HindIII	AAGCTT	15	e"21226, 8985, 6178, 4430, 3687, 3037, 2084, 1813, 1637, 1274, 1177, 875, 732, 641, 548	e"57098	23	23	22
14	XhoI	CTCGAG	3	e"21226, 15692, 6178	NA	1 (No cut)	1 (No cut)	1 (No cut)
15	PstI	CTGCAG	10	e"21226, 13195, 6608, 5831, 5000, 3876, 3619, 3095, 1921, 1435	e"64580	1 (No cut)	1 (No cut)	1 (No cut)
16	EcoRI	GAATTC	2	e"21226, 13195	NA	6	6	6
17	BamHI	GGATCC	1 (No cut)	e"21226	NA	3	3	3
18	Eco31I	5'...G G T C T C (N) "1...3'3' ...C C A G A G (N) '1...5' AGCT	1(No cut)	e"21226	NA	9	9	9
19	AluI	AGCT	Uncountable	—	NA	159	159	149

information and development of genetic fingerprints for identification and authentication of organism. In the present study, the genetic fingerprinting of phage DNA was developed by restriction digestion using 19 restriction endonucleases of various recognition types, frequent and rare cutters ranged from tetra cutter to octacutter viz., *Bgl*II, *Sall*, *Sac*I, *Ava*I, *Apal*, *Not*I, *Eco*RII, *Eco*RV, *Kpn*I, *Nco*I, *Xba*I, *Bsp*119I (*Bst*BI), *Hind*III, *Xho*I, *Pst*I, *Eco*RI, *Bam*HI, *Eco*31I (*Bsa*I) and *Alu*I. After 3 hours of digestion at 37°C the digested product were separated on 0.7% agarose gel at 3mV/cm for 6-9 hours along with high and medium range ladder/ molecular marker and the size of bands/ fragments were determined based on the marker. The gel images were analysed using Image lab software (Figure 4).

Out of 19 restriction endonucleases used, three enzyme namely; *Eco*31I (*Bsa*I), *Apal* and *Bam*HI were unable to digested the phage genome resulting no fragment was observed in gel except phage genome, which indicated the lack of recognition sites of these enzymes in the phage genome. However, the ϕ Ld phage DNA was found to be sensitive to rest of the restriction enzymes used yielding number of fragments ranged from 2-21 or more in case of tetra cutter (*Alu*I). The restriction enzymes namely, *Eco*RI and *Not*I

produced only two fragment while *Xho*I, *Xba*I and *Bsp*119I (*Bst*BI) produced 3, 4 and five fragments, respectively. The result indicated the presence of having only few (one to four) restriction sites for these endonucleases in the phage genome. While rest other endonucleases have many restriction sites in phage DNA thus yielded many fragments upon digestion viz., 10 fragments by *Sall*, *Sac*I, *Kpn*I and *Pst*I; 15 and 16 fragment by *Hind*III and *Ava*I respectively; 18 fragments by *Eco*RV and *Nco*I; 21 fragments by *Bgl*II and *Eco*RII; and uncountable number of fragments by *Alu*I. The restriction results with the endonucleases were highly reproducible and thus can be used for fingerprinting and identification of the phage. The ϕ Ld phage genome size estimated from restriction fragment length polymorphism (RFLP) pattern was ranging approximately between 56kbp to 81kbp (Figure 4 and Table 1). RFLP has been routinely used to characterize the phage genome (Barrangou *et al.*, 2002; Shivu *et al.*, 2007; Kumari *et al.*, 2009; Zhu *et al.*, 2009; Flores *et al.*, 2012).

Comparison of RFLP fingerprints of phage ϕ Ld with reference phage Tbilisi and Weybridge

DNA restriction digestion patterns were compared to *in silico* genome digestion analysis of selected phages. Brucellaphage Tb was isolated from manure in Tbilisi, Georgia in 1955 while phage

Table 2. Genomic fingerprinting of Brucellaphage ϕ Ld by RAPD analysis

S. No.	Primer Name	Primer Sequence 5'-3'	No. of bands	Details of band size
1	OPN-02	ACCAGGGGCA	5	1747, 1427, 1030, 800 and 453
2	OPN-04	GACCGACCCA	2	1470 and 250
3	OPN-06	GAGACGCACA	6	2979, 2281, 1713, 1506, 910 and 754
4	OPN-07	CAGCCCAGAG	Nil	Nil
5	OPN-10	ACAACCTGGGG	7	1188, 966, 837, 683, 580, 496 and 322
6	OPN-11	TCGCCGCAAA	Nil	Nil
7	OPN-15	CAGCGACTGT	4	1255, 581, 491 and 361
8	OPN-18	GGTGAGGTCA	4	2793, 1900, 1529 and 777
9	OPQ-01	GGGACGATGG	6	2835, 2469, 1552, 1385, 1035 and 785
10	OPQ-02	TCTGTCTGGTC	Nil	Nil
11	OPQ-03	GGTCACCTCA	Nil	Nil
12	OPQ-04	AGTGCGCTGA	6	2518, 1881, 1638, 1413, 1082 and 747
13	OPQ-06	GAGCGCCTTG	8	2457, 2046, 1909, 1351, 1299, 1109, 673 and 445
14	OPQ-10	TGTGCCCGAA	3	2067, 1137 and 1015
15	OPA-11	CAATCGCCGT	1	1365
16	OPA-12	TCGGCGATAG	Nil	Nil
17	OPA-13	CAGCACCCAC	Nil	Nil
18	OPA-14	TCTGTGCTGG	1	1891

Wb was isolated from *B. suis* culture in Weybridge, England in 1973. Genomes of phage Tb and Wb have been previously sequenced and deposited at GenBank, accession number: KC556897 and KC556898 respectively (Farlow *et al.*, 2014). The

Tb and Wb genomes consist of 41,148 bp and 38253 bp respectively. The nucleotide sequences of both phages were downloaded from genbank and *in silico* restriction pattern was generated using NEB cutter.

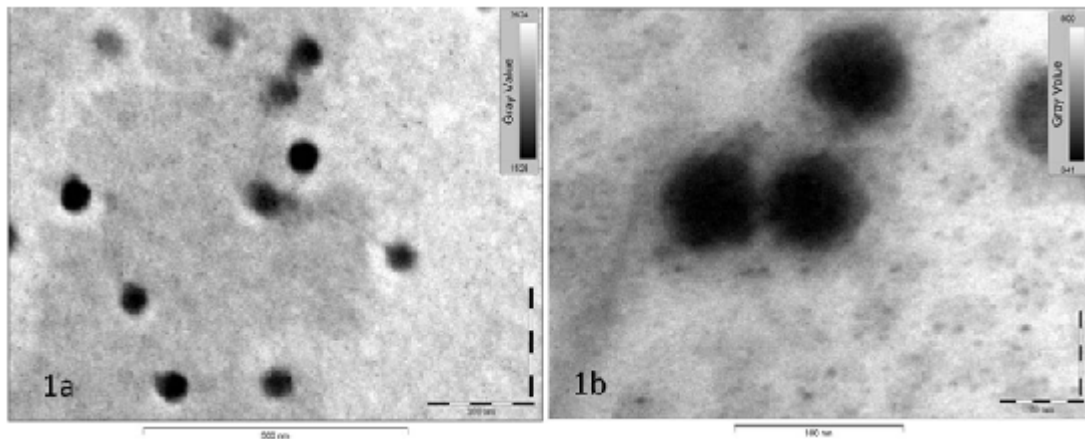
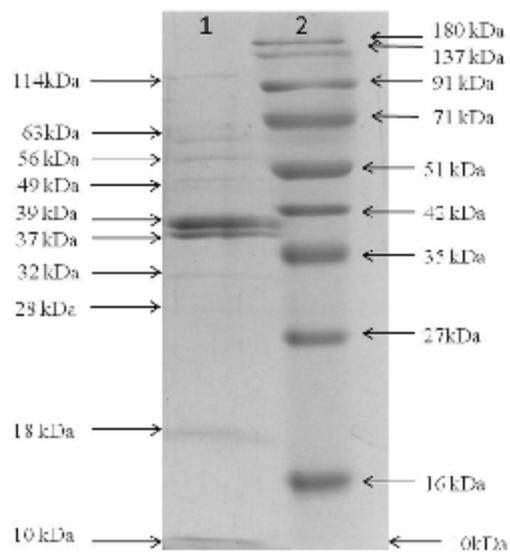
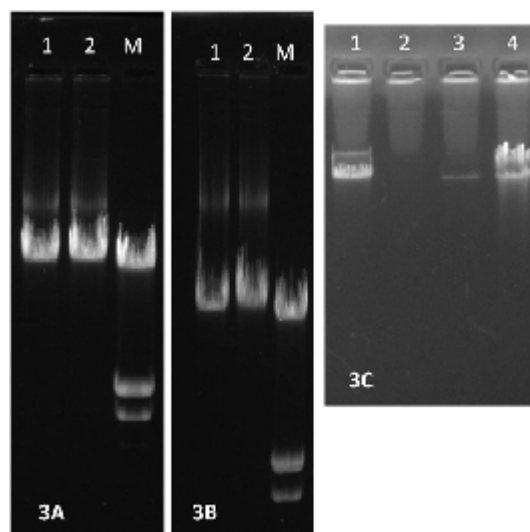


Fig. 1. (a & b): Electron micrograph image of phage ϕ Ld showing icosahedral head and short tail



Lane 1. Phage proteins
Lane 2. Protein Marker Puregene

Fig. 2. SDS-PAGE pattern of structural proteins of Brucellaphage ϕ Ld



(3A) and (3B): Agarose gel migration pattern of nucleic acid of Brucellaphage ϕ Ld after 6 hours and 9 hours of electrophoresis respectively, where (1) lambda DNA uncut; (2) ϕ Ld nucleic acid; and (M) Lambda EcoRI + HindIII digested marker.

(3C): Agarose gel electrophoresis of phage nucleic acid. (1) Undigested; (2) digested with DNaseI; (3) Digested with Rnase A and (4) Digested with S1 Nuclease.

Fig. 3. Agarose gel electrophoresis of ϕ Ld phage nucleic acid.

The restriction patterns of both the phage were compared showing significant difference in sequences of the phage and reference phages (Table 1). The reference Tb and Wb phage does not have restriction site for *NotI*, *XhoI* and *PstI* while the ϕ Ld phage has one, 2 and 9 restriction sites respectively for these endonucleases. The endonuclease *ApaI*, *BamHI* and *Eco31I* were unable to digest the ϕ Ld phage DNA while

restriction sites for these enzymes were present in the Tb and Wb phage DNA. Similarly, there is significant difference in the digestion pattern produced with other endonucleases between the reference phages and ϕ Ld phage. These differences might be due to variation in genomic sequences of the phages as a consequence of integration or deletion of unusual bases in the viral DNA or repetition of genomic fragments.

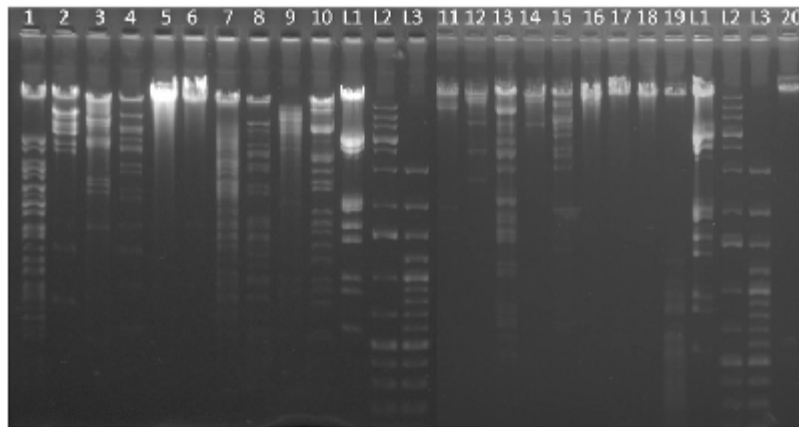


Fig. 4. Agarose gel electrophoresis of ϕ Ld Brucellaphage DNA digested with restriction endonucleases. Lanes are (1) BglI; (2) SalI; (3) SacI; (4) Aval; (5) ApaI; (6) NotI; (7) EcoRII; (8) EcoRV; (9) KpnI; (10) NcoI; (11) XbaI; (12) Bsp119I (BstBI); (13) HindIII; (14) XhoI; (15) PstI; (16) EcoRI; (17) BamHI; (18) Eco31I (BsaI); (19) AluI treated Brucellaphage DNA; (20) Undigested/untreated DNA; (L1) Lambda HindIII/EcoRI digested molecular size marker; (L2) Molecular size marker one Kbp plus and (L3) Molecular size marker 100 bp plus.

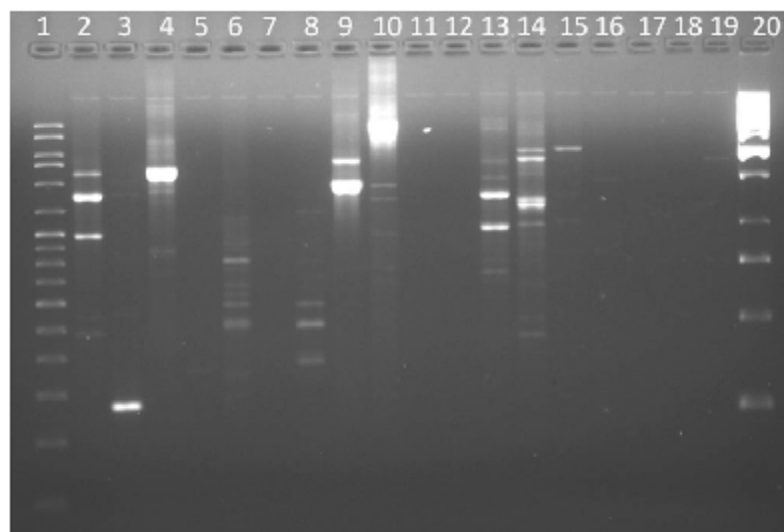


Fig. 5. Agarose gel electrophoresis RAPD-PCR products of ϕ Ld Brucellaphage. Lane are (1) 100bp ladder; (2) OPN 02; (3) OPN 04; (4) OPN 06; (5) OPN 07; (6) OPN 10; (7) OPN 11; (8) OPN 15; (9) OPN 18; (10) OPQ 01; (11) OPQ 02; (12) OPQ 03; (13) OPQ 04; (14) OPQ 06; (15) OPQ 10; (16) OPA 11; (17) OPA 12; (18) OPA 13; (19) OPA 14 and (20) 1 Kb ladder.

Genomic fingerprinting by RAPD PCR

RAPD-based PCR do not require sequence information for primer design. To generate a fingerprint of the ϕ Ld phage in the present study, RAPD PCR was performed with the phage DNA using 18 different primers (Table 2), out of which six primers viz., OPN07, OPN11, OPQ02, OPQ03, OPN12 and OPN13 were unable to amplify the phage DNA due to absence of compatible primer binding site in the genomic DNA sequence. The phage DNA was amplified by 12 primers and each generated distinct banding pattern ranged from one to eight fragments with amplicons ranging in size from approximately 250 to 3000 bp (Figure 5 and Table 2). The primer OPA-11, OPA-14 and OPN-04 produced only one to two amplicon. While the primer OPN-10 and OPQ-06 produced 7 and 8 amplicons respectively. The number of bands and banding patterns produced by each primer was unique and highly reproducible. These RAPD fingerprints can be used for identification, typing of the phage and discriminating between closely related brucellaphage strains.

DNA polymorphisms amplified with the random primers of ten or more nucleotides in length could able to find the primer binding site and amplified the fragments which are separated on gel electrophoresis and produced the genetic fingerprint. The PCR based RAPD technique required no prior genomic information of organism and needs single primer and minute quantity of template DNA for amplification. This technique has proven to be very practicable for development of genetic fingerprinting and assessment of genetic diversity and also useful in providing valuable information on the relatedness and to establish identity of the phages. Results obtained in this study confirmed these earlier observations that RAPD analysis can be used as rapid method for identification, typing and discrimination of closely related phages.

RAPD typing had been applied successfully to the genetic fingerprinting of man, plants, fungi, bacteria and viruses. RAPD-PCR using purified DNA has also been used to generate fingerprints and assess the genetic diversity of *Vibriophages* (Comeau *et al.*, 2006; Shivu *et al.*, 2007) and phages infecting *Escherichia coli* (Dini and Urraza, 2010), *Pseudomonas aeruginosa* (Li *et al.*, 2010), *Leuconostoc fallax* (Barrangou *et al.*,

2002) and *Brucella abortus* (Zhu *et al.*, 2009). Gutierrez *et al.*, 2011 evaluated RAPD-PCR technique to produce unique and reproducible band patterns from 26 different bacteriophages infecting *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Lactococcus lactis*, *Escherichia coli*, *Streptococcus thermophilus*, *Bacillus subtilis* and *Lactobacillus casei* bacterial strains. These results support the use of RAPD-PCR for quick typing of phage isolates and preliminary assessment of their genetic diversity bypassing tedious DNA purification protocols and previous knowledge of their sequence.

CONCLUSIONS

In this study, the brucellaphage isolated in India was characterized for its morphology, protein profiling and genomic fingerprinting by RFLP and RAPD. The studied phage resembles the reference brucellaphage in terms of morphological characteristics and structural protein profile. There was significant difference in genomic sequence of isolated phage and the known brucellaphages as evident by the variations in the sensitivity to endonucleases and banding patterns. These relationships deserve further exploration and emphasize the need of full genome sequencing of the Indian isolate of brucellaphage to decipher genomic and proteomic information.

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