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Characterization of Brucellaphage using RAPD and microsatellite repeat marker

Lata Jain[1]*, Mayank Rawat[1], Vinay Kumar[2] and Deepti Chachra[3]

[1]Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar - 243 122, India.
[2]Directorate of Medicinal and Aromatic Plant Research, Boriavi, Anand, Gujarat - 387310, India.
[3]Department of Veterinary Microbiology, College of Veterinary And Animal Sciences, Guru Angad Dev Veterinary and Animal Science University, Ludhiana (Punjab)-India.

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Brucellosis is an important zoonotic infection of worldwide significance. In this study the recently isolated lytic brucellaphage 'ΦLd' against Brucella abortus was characterized using random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers. Out of 18 RAPD primers used, 15 primers were able to generate distinct finger prints / amplicons ranged from two to twelve fragments. The RAPD finger prints produced were distinct and highly reproducible. A total of 18 microsatellite repeat (ISSR) markers used in the study had revealed that the phage genome consists of di-nucleotide repeats of AG, TG and CT and tri-nucleotide repeat of AGC and ACG. The RAPD and microsatellite fingerprinting has generated the basic information about the genomic composition of the Indian isolate of Brucellaphage which could be useful in the identification and discrimination of new phage and high resolution brucellaphage diversity studies.

Key words: Brucellaphage, Random amplified polymorphic DNA, ISSR marker.

Brucellosis is a globally spread disease of zoonotic, public health and economic importance worldwide infecting animals and humans in the form of reproductive and severe systemic disease (Corbel, 1997). Abortion, stillbirths, neonatal deaths and infertility decreased milk production and repeat breading are some of the clinical manifestations of the disease leading to temporary or permanent infertility in infected livestock (Moreno *et al.*, 2002; Verma, 2013). It is prevalent in most of the developing countries and is caused by Gram negative facultative intracellular bacterial organisms of the genus *Brucella*. In India, brucellosis was first recognized in 1942 and now has been reported from almost all the states (Renukaradhya *et al*., 2002).

The bacteriophages that infect *Brucella* species are called Brucellaphages. Phages are viruses that infect and multiply inside the bacteria. They are non living entity outside the host cell and require host cell machinery for replication and other metabolic processes with two types of life cycles, lytic and lysogenic (Carlton, 1999; Harper *et al.*, 2010).

Existence of bacteriophages against *Brucella* has been reported by researchers since 1960s with the discovery of phage Tibilisi (Pickett and Nelson, 1951; Drozhevkina, 1956). All the brucellaphages that have been described to date belong to the family *Podoviridae* of order *Caudovirales* (Ackermann, 2007). These are linear non-enveloped icosahedral phages with short noncontractile tails and linear double-stranded DNA viruses.

* To whom all correspondence should be addressed.
E-mail: jain.lata59@rediffmail.com
jain.lata59@gmail.com

Brucellaphages have been used in speciation and biotyping of *Brucellae*, but little is known about the brucellaphage genomics. In the recent years, molecular characterization of reference Tbilisi phage DNA (Zhu *et al.,* 2009), genetic finger printing using RFLP and RAPD (Jain *et al.*, 2014), 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 (Farlow *et al.,* 2014) had been reported.

In the present study, the brucellaphage 'φLd' against *Brucella abortus* S 19 isolated at GADVASU, Ludhiana, India (Chachra *et al.*, 2012; Pandey *et al.,* 2013) was characterized for molecular profiling by random amplified polymorphic DNA and inter simple sequence repeat microsatellite marker to facilitate its wider use in various diagnostic and immunotherapeutic applications.

MATERIALS AND METHODS

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 and propagation of Brucellaphage

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 $MgSO_4.7H_2O$, 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 abortus* (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.

Isolation of Nucleic acid

Phage stock having titre $\leq 10^9$ 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 treated with DNase I and RNase A at 37°C for 1 hour to remove 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 *Eco*RI + *Hind*III digested marker.

Genomic fingerprinting by RAPD analysis

RAPD was carried out as per protocol described by Williams *et al.,* 1990 using 18 random primers of series N, P and Q from Operon Technologies (Alameda, USA). PCR with single primer was carried out in a final volume of 20µl containing 2.0 µl of 10x assay buffer (Tris-Cl; pH 9.0, KCl and gelatin) 1.5 mM $MgCl_2$, 200µM 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 94ºC for 4 min followed by 40 cycles each of 1 min at 94ºC, 1 min at 37 ºC, 2 min at 72 ºC.

The final step consisted of one cycle of 7 min at 72ºC for complete polymerization. After completion of the PCR, 2.5 µl of 6X loading dye (MBI Fermentas Inc. USA) was added to the amplified products and were electrophorsed in a 1.5% (w/v) agarose (MBI Fermentas 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, California US). The sizes of the amplification products were estimated by comparing them to standard DNA ladder (Gene Ruler 100 bp plus DNA ladder Banglore genei and 1 Kb DNA ladder; MBI Fermentas Inc. USA).

Genomic fingerprinting by ISSR markers

A set of 18 numbers of Inter simple sequence repeat markers primers were used for molecular fingerprinting of *Brucellaphage* (φLd) to detect internal repeats of two nucleotides and three nucleotides sequence. PCR with single primer was carried out in a final volume of 20µl containing 2.0 µl of 10x assay buffer (Tris-Cl; pH 9.0, KCl and gelatin) 25 mM $MgCl_2$, 100 mM of each dNTPs (MBI Fermentas Inc. USA) 20 ng 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 94ºC for 4 min followed by 40 cycles followed by 40 cycles each of 1 min at 94ºC, 1 min (optimized temperature for each primer), 2 min at 72 ºC. The final step consisted of one cycle of 7 min at 72ºC for complete polymerization. After completion of the PCR, 2.5 µl of 6X loading dye (MBI Fermentas Inc. USA) was added to the amplified products and were electrophorsed in a 1.5% (w/v) agarose (MBI Fermentas Inc. USA) gels with 1x TAE buffer, stained with ethidium bromide and visualised under UV light. Gel photographs were scanned through by using Molecular Imager Gel Doc XR+ (Biorad, California US). The sizes of the amplification products were estimated by comparing them to standard DNA ladder (Gene Ruler 100 bp plus DNA ladder banglore genei and 1 Kb DNA ladder; MBI Fermentas Inc. USA).

Table 1. Genomic fingerprinting of Brucellaphage ΦLd by RAPD analysis

S. No.	Primer Name	Primer Sequence 5'-3'	No. of bands	Details of band size
1	OPN-01	CTCACGTTGG	5	2213, 1976, 1718, 1530, 1334
2	OPN-03	GGTACTCCCC	Nil	No amplification (NA)
3	OPN-05	ACTGAACGCC	9	2793, 2624, 2495, 2127, 1662, 1407, 931, 703, 400
4	OPN-08	ACCTCAGCTC	3	1273, 900, 374
5	OPN-09	TGCCGGCTTG	8	3624, 1963, 1718, 1343, 1231, 842, 542, 152
6	OPN-12	CACAGACACC	4	2580, 1963, 645, 334
7	OPN-13	AGCGTCACTC	10	3979, 2546, 2199, 1989, 1788, 1544, 1334, 1085, 762, 487
8	OPN-14	TCGTGCGGGT	9	2546, 1765, 1334, 1092, 982, 860, 722, 620, 396
9	OPN-16	AAGCGACCTG	7	3346, 2832, 1765, 1608, 1407, 1022, 676
10	OPN-17	CATTGGGGAG	2	1022 and 485
11	OPN-19	GTCCGTACTG	11	2155, 1963, 1753, 1586, 1325, 1099, 968, 876, 690, 600 and 512
12	OPN-20	GGTGCTCCGT	12	2057, 1730, 1565, 1273, 1152, 865, 752, 649, 564, 468, 376 and 300
13	OPQ-07	CCCCGATGGT	11	2988, 2495, 2084, 1899, 1555, 1264, 1114, 859, 772, 658 and 500
14	OPQ-11	TCTCCGCAAC	6	2213, 1861, 1718, 620, 420 and 305
15	OPQ-12	AGTAGGGCAC	6	3600, 2479, 1334, 1175, 995 and 658
16	OPQ-20	TCGCCCAGTC	12	3576, 2615, 2335, 2098, 1640, 1273, 1085, 918, 831, 487, 445 and 330
17	OPP-01	GTAGCACTCC	Nil	NA
18	OPP-02	TCGGCACGCA	Nil	NA

Table 2. Characterization of Brucellaphage ΦLd by microsatellite based- ISSR markers

Name of the Primers	Primer sequences (5'- 3')	Primer length (bp)	Annealing Temperature	No. of bands Amplified	Detail and size of amplified (Bands) products
(CT)9G	CTCTCTCTCTCTCTCTCTG	19	55	4	1453, 1082, 751 and 505
(AG)8C	AGAGAGAGAGAGAGAGC	17	52	2	1337 and 994
(CA)8AT	CACACACACACACACAAT	18	47	Nil	Nil
(TG)8G	TGTGTGTGTGTGTGTGG	17	48	4	1490, 1165, 1052 and 818
(TG)8C	TGTGTGTGTGTGTGTGC	17	48	1	791
(AG)8G	AGAGAGAGAGAGAGAGG	17	50	8	1924, 1752, 1372, 1006, 949, 814, 633 and 516
(AG)8R	AGAGAGAGAGAGAGAGA	17	50	4	1911, 1337, 1133 and 994
(AGC)6G	AGCAGCAGCAGCAGCAGCG	19	57	12	2000, 1425, 1247, 1165, 1006, 880, 800, 682, 643, 503, 439 and 260
(AGC)6AG	AGCAGCAGCAGCAGCAGCAG	20	58	10	1435, 1152, 965, 755, 693, 600, 541, 458, 346 and 271
(CA)8AC	CACACACACACACACAAC	18	50	Nil	Nil
(CA)8GT	CACACACACACACACAGT	18	48	Nil	Nil
(AG)8YT	AGAGAGAGAGAGAGAGGT	18	48	Nil	Nil
(AC)8GA	ACACACACACACACACGA	18	50	Nil	Nil
(CT)8AG	CTCTCTCTCTCTCTCTAG	18	48	Nil	Nil
(CT)8AC	CTCTCTCTCTCTCTCTAC	18	50	3	1279, 543 and 330
(CA)8GT	CACACACACACACACAGT	18	48	Nil	Nil
(ACG)6TG	ACGACGACGACGACGACGTG	20	59	11	1949, 1082, 943, 870, 733, 640, 600, 549, 424, 332 and 278
(TC)8GA	TCTCTCTCTCTCTCTCGA	18	50	Nil	Nil

Data analysis

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

RESULTS AND DISCUSSION

Genomic fingerprinting by RAPD PCR

RAPD-based PCR do not require sequence information for primer design. In the present study, RAPD PCR was performed with the phage DNA using 18 different primers to generate fingerprints of the ΦLd phage (Table 1), out of which three primers viz., OPN03, OPP01 and OPP02 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 15 primers and each generated distinct banding pattern ranged from two to twelve fragments with amplicons ranging in size from approximately 152 to 3979 bp (Figure 1 and Table 1). The primer OPN-17 and OPN-08 produced only 2 to 3 amplicons. While the primer OPN-12, OPN-01, OPQ-11 OPQ-12, OPN-16 and OPN-09 produced 4 to 8 amplicons. The primers OPN-05, OPN-14, OPN-13, OPN-19, OPQ-07, OPN-20 and OPQ-20 produced 9 to 12 amplicons. 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.

RAPD analysis of phage DNA provided a simple and reproducible method for identification and differentiation of bacteriophages. RAPD markers are well suited for genetic fingerprinting and provide an efficient assay for phage polymorphisms. This technique required no prior genomic information of organism and needs single primer and minute quantity of template DNA for amplification.

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-PCR has been used to generate fingerprints and assess the genetic diversity of phages infecting *Brucella abortus* (Zhu *et al.,* 2009; Jain *et al.,* 2014), *Pseudomonas aeruginosa* (Li *et al.,* 2010), *Bacillus subtilis* (Marie, 2013), *Oenococcus oeni* (Doria *et al.,* 2013), *Leuconostoc fallax* (Barrangou *et al.,* 2002), *Salmonella* Typhimurium and *S.* Enteritidis (Fiorentin *et al.,* 2004), *Escherichia coli* (Dini and Urraza, 2010), and *Vibriophages* (Comeau *et al.,* 2006; Shivu *et al.,* 2007). Gutierrez *et al.,* 2011 evaluated RAPD-PCR technique to produce unique and reproducible

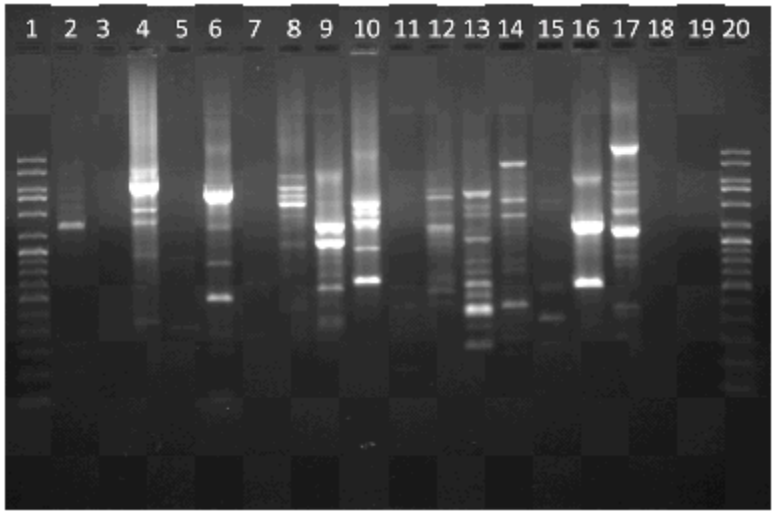

Fig. 1. Agarose gel electrophoresis of RAPD-PCR products of Brucellaphage ΦLd. Lanes are (1) 100bp ladder Banglore Genei; (2) OPN 01; (3) OPN 03; (4) OPN 05; (5) OPN 08; (6) OPN 9; (7) OPN 12; (8) OPN 13; (9) OPN 14; (10) OPN 16; (11) OPN 17; (12) OPN 19; (13) OPN 20; (14) OPQ 07; (15) OPQ 11; (16) OPQ 12; (17) OPQ 20; (18) OPP 1; (19) OPP 2 and (20) 100bp ladder Banglore Genei

band patterns from 26 different bacteriophages infecting *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Lactococcus lactis*, *Escherichia coli*, *Streptococcus thermophilus*, *Bacillus subtilis* and *Lactobacillus casei* bacterial strains. The results of all these studies 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.

Genomic fingerprinting by ISSR markers

To characterize and develop genetic fingerprints of the φLd phage using PCR based microsatellite markers; a set eighteen Inter Simple Sequence Repeat (ISSR) markers anchored at 3' end were used. Of which eight primers *viz.*; (CA)8AT, (CA)8GT, (AG)8YT, (AC)8GA, (CT)8AG, (CA)8GT and (TC)8GA could not amplified any amplicons in the ΦLd phage genome. The lack of amplification may be due to absence of compatible primer binding site for particular repeat motifs or higher specificity of primers at 3' end due to anchored two nucleotides.

The genetic fingerprinting of the ΦLd phage genome developed by 10 microsatellite markers produced distinct banding pattern, the number of amplicons amplified in ΦLd phage genome varied from one to twelve produced by (TG)8C and (AGC)6G primers, respectively. However, the size of amplicons varied from 250 to 3000 bp in length. It was found that the phage genome was having di-nucleotide repeats of AG, TG and CT. While, the phage genome didn't show the presence of di-nucleotide repeat of CA. Similarly, the phage genome was having tri-nucleotide repeat of AGC and ACG. These microsatellite based fingerprints could be deployed for identification, typing of the phage and discriminating between closely related Brucellaphage strains (Table 2 and Figure 2).

Among the microsatellite markers used, the tri-nucleotide repeats namely; (AGC)6G, (AGC)6AG and (ACG)6TG amplified the highest number of amplicons 12, 10 and 11 bands, respectively. Similarly, number of bands amplified by di-nucleotide repeats (AG)8G, (TG)8G, (CT)9G, (AG)8R, (CT)8AC, (AG)8C and (TG)8C varied 8, 4, 4, 4, 3, 2 and 1 bands, respectively.

Microsatellites are short tandem repeats of 1 to 6 bp and are the most mutable DNA sequences found in eukaryote, prokaryote, and some virus genomes, particularly in intergenic regions and introns (Tautz and Renz, 1984; Field and Wills, 1996). Apart from all these, microsatellites are known to be highly polymorphic by nature as they gain/loss repeat units (motifs) in course of time, thus, making them highly important in the studies of genome evolution (Jarne and Lagoda, 1996). However, the knowledge about the microsatellite distribution remains largely enigmatic in viruses yet is crucial for understanding

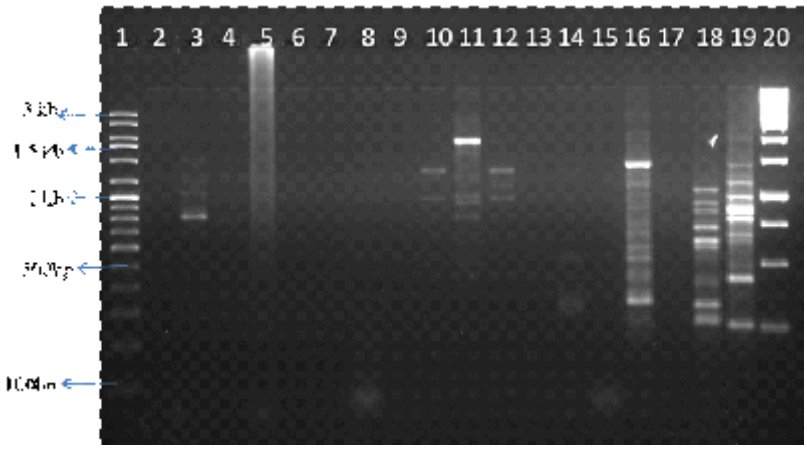

Fig. 2. Agarose gel electrophoresis of ISSR marker based PCR products. Lane details are (1) 100bp ladder Banglore Genei; (2) IS-3; (3) IS-4; (4) IS-5; (5) IS-12; (6) IS-13; (7) IS-14; (8) IS-15; (9) IS-17; (10) IS-2; (11) IS-6; (12) IS-7; (13) IS-11; (14) IS-16; (15) IS-20; (16) IS-10; (17) IS-1; (18) IS-19; (19) IS-8; and (20) 1Kb ladder Fermentas # SM1163

instability of viral genomes. Though, the genome of Indian isolate of brucellaphage ΦLd is not yet sequenced, characterization of ΦLd using microsatellite based- ISSR markers, shed light on the presence of microsatellite repeats motifs and their types in the phage genome. It was found that in φLd genome among the dinucleotide repeats (AG) and TG are more prevalent followed by CA and CT and in tri-nucleotide repeats (AGC) is prevalent type of repeat.

Studies in certain viral genomes have also shown that polymorphism does exist among the viruses and numerous polymorphic microsatellites were detected in human cytomegalovirus (HCMV), herpes simplex virus type 1 (HSV-1), Ostreid herpesvirus 1 (OsHV-1) and hepatitis C genomes (Davis *et al.*, 1999; Deback *et al.*, 2009; Segarra *et al.*, 2010; Chena *et al.*, 2011) and proven to be useful as markers in epidemiological and virulence studies (Hood *et al.*, 1996). Studies also show that microsatellites play an important role in the evolution of new virus strains (Deback *et al.*, 2009). The microsatellite fingerprints revealed the basic information about the composition and type of microsatellite repeat motif in the Indian isolate of Brucellaphage genome and the genetic fingerprints developed during the study could be useful in the identification and discrimination of new stains/ phage on the basis of its banding pattern.

CONCLUSIONS

In this study, the brucellaphage isolated in India was characterized by RAPD and ISSR markers. 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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