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Isolation and Characterization of BpL1, a Broad Acting Lytic Bacteriophage against *Brucella*

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A B S T R A C T

In view of the ever increasing antimicrobial resistance, bacteriophages are promising alternatives for treatment of bacterial infections. Brucellosis is an important zoonotic disease for which currently there is no satisfactory treatment. We have isolated a new broad acting lytic brucellaphage (BpL1) from the sewage of a dairy farm. The phage lysed all the 12 *Brucella abortus* field isolates, *B. abortus* strain 99 and *Brucella melitensis* but did not lyse any of the heterologous species tested viz. *Staphylococcus aureus, Pasteurella multocida, Escherichia coli,* and *Salmonella* species. Streaking the lysis plaques on *Brucella* lawn gave clear lytic zones along the streak lines. The plaques were circular with a diameter of 0.5- 3.0 mm. At a concentration of 10^{-4} the phage count was 4.5×10^{6} plaques per ml. It was a tailed phage with icosahedral head (62.2 nm in diameter and 73.71 nm in length), and the head to tail length was 229.21 nm. The phage belonged to the order Caudovirales and family *Siphoviridae*. It was inactivated within one hour at 55°C and within 4 h at -20°C. Treatment at pH 2 for 4 h and at pH 4 for 12 h inactivated it. It was also inactivated after 4 h exposure to sunlight, and within 4 min. by UV light. Chloroform and Sodium Dodecyl Sulfate inactivated it within 15 min. Lysozyme inactivated it within 1 hour whereas RNase treatment did not affect its activity.

Introduction

Brucellosis is a highly contagious and important zoonotic disease caused by different species of the genus *Brucella.* The *Brucella* organisms are pathogenic for a wide variety of animals such as swine, cattle, goat, sheep, and dogs and also for humans (Mathur *et al.*, 2007). In animals, brucellosis mainly affects reproduction and fertility, reduces the survival of newborns, and diminishes milk yield. Outbreak of Brucellosis in animals is characterized by abortions during the last trimester of gestation. Brucellosis is endemic in India and is prevalent in all parts of the country. Currently, no effective and affordable treatment is available for Brucellosis in large animals. Antibiotic resistance has been detected in *Brucella* organisms also (Gupta and Saxena, 2017). The widely used S-19 vaccine has not been successful in controlling Bucellosis since infection in some vaccinated animals has also been observed (Mohan *et al.*, 2016). A bacteriophage is a virus that specifically infects and lyses its host bacterium. This unique characteristic can be exploited for therapy of infections due to antibiotic

resistant bacteria. We report here the isolation of a new Brucellaphage from the habitat of *Brucella* infected animals i.e. sheds of infected cattle.

Materials and Methods

Isolation, identification and characterization of *Brucella abortus*

Brucella abortus vaccine strain S19, *Brucella abortus* S99, *B. melitensis* Rev1 and *B. melitensis* were grown on *Brucella* selective agar (HiMedia) and incubated at 37^{o}C aerobically. *B. abortus* from field samples were isolated on Brucella selective agar (Gupta and Saxena, 2017). Samples comprising of aborted foetal stomach contents, placenta and cotyledons and vaginal and uterine secretions from cattle and buffaloes with a history of abortions were collected and were subjected to isolation. *Brucella* isolates were identified on the basis of cultural, morphological and biochemical characteristics. DNA extraction was done as per the standard protocols of Sambrook and Russell (2001). Molecular characterization of the field isolates was done by polymerase chain reaction (PCR) as per the method of Romero *et al.,* (1995).

Isolation of bacteriophage against *Brucella abortus*

Agar overlay technique was used to isolate bacteriophage against *Brucella abortus* (Adams 1959, Chilamban *et al.,* 2004). A set of available heterologous species of bacteria of veterinary importance viz. *Pasteurella multocida B: 2, E.coli, Staphylococcus, Streptococcus, Salmonella Dublin, Micrococcus* and *Pseudomonas* species were used.

A total of seven sewage samples were collected from the drain of a dairy farm in Ludhiana at different times and processed for the isolation of phage. In brief, to the 50 ml double strength NZCYM broth (Life Technologies) 40 ml sewage supernatant and 10 ml of broth culture of *B. abortus* in exponential growth were added and incubated on rotary shaker for 10 days at 37^{o}C. Out of this incubated sewage containing bacteria cocktail, 10 ml of supernatant was taken every day and centrifuged at 8000g for 15 min. to collect the supernatant which was passed through 0.22µm PVDF filter (Axiva) and the filtrate was aseptically collected and stored at 4^{o}C till further use and was designated as Bacteria Free Filtrate (BFF). Equal quantities (100 µl) of BFF and overnight broth culture of *B. abortus* were mixed in 0.75% NZCYM agar (maintained at 45^{o}C in a dry bath) and was spread evenly over 1.5% NZCYM agar containing BSM agar. The soft agar was allowed to solidify and the plates were incubated at 37^{o}C for 48-72 h to observe plaques.

Elution of brucellaphage

The plaques were picked using a straight wire loop and were streaked horizontally and then vertically on a hardened NZCYM + BSM plate overlaid with semisolid NZCYM agar containing the indicator strain. The plate was incubated at 37^{o}C for 18h to observe plaques along the lines. SM buffer (2ml) was poured over the agar and the agar was disturbed with the wire loop to release the phages from the semisolid agar. This SM buffer was then collected and centrifuged at 5000g to remove the agar and then the supernatant was filtered through 0.22 µm filters to remove the bacteria and elute the phage in SM buffer.

Effect of varied temperatures on the brucellaphage

100µl of brucellaphage (10^{6}pfu/ml) was subjected to temperatures of -20^{o}C, 4^{o}C,

37°C, 50°C, 70°C and 100°C for a period of 20 min. Freshly grown *Brucella abortus S19* culture (200µl) was added in cooled molten semisolid NZCYM and plated on BSM+NZCYM plates. These plates were incubated aerobically at 37°C for 48 to 72 h. Any change in pfu was observed.

Effect of light on the brucellaphage

100µl of brucellaphage (10^6pfu/ml) was subjected to normal fluorescent tube light, sunlight and UV light for a period of 15 min to 90 min. 200µl of freshly grown *Brucella abortus S19* culture was added in cooled molten semisolid NZCYM and plated on BSM+NZCYM plates. The plates were incubated aerobically at 37°C for 48 - 72 h. It was observed for any change in pfu.

Effect of enzymes on the brucellaphage

100µl of brucellaphage (10^6pfu/ml) and 100 µl of enzymes *viz*. proteinase K (20mg/ml), trypsin (250µg/ml), lysozyme (20mg/ml) and RNAse (10mg/ml) were incubated for 15 min. 200µl of freshly grown *Brucella abortus S19* culture was added in cooled molten semisolid NZCYM and plated on BSM+NZCYM plates. These plates were incubated aerobically at 37°C for 48 to 72 h. Any change in pfu was noted down.

Effect of SDS, normal saline and EDTA on the brucellaphage

100µl of brucellaphage (10^6pfu/ml) was subjected to treatment with equal volume of 10% Sodium dodecyl Sulfate (SDS), Normal Saline Solution (NSS) and EDTA (0.01M) for a period of 15 min to 3h. Any change in pfu was observed by adding 200µl of freshly grown *Brucella abortus S19* culture in cooled molten semisolid NZCYM and plated on BSM+NZCYM plates. These plates were incubated aerobically at 37°C for 48 to 72 h.

Effect of varied pH on the survivability of bacteriophage

100µl of brucellaphage (10^6pfu/ml) was observed for the change in pfu count in different pH ranges of 3, 5, 7 and 9 for periods of 30 min and 60 min exposure time. Any change in pfu was observed by adding 200µl of freshly grown *Brucella* culture in cooled molten semisolid NZCYM and plated on BSM+NZCYM plates. These plates were incubated aerobically at 37°C for 48 to 72 h.

Results and Discussion

Isolation of brucellaphage

Out of the total 36 sewage samples, brucellaphage could be isolated from one sample. Streaking the plaques on *Brucella* lawn gave clear zones along the streak lines (Fig. 1).

At a concentration of 10^{-4} the phage count was 4.5×10^6 plaques per ml. We propose the name "Brucellaphage Ludhiana 1 (BpL1)" for this new phage.

Heterogeneity test for brucellaphage

The isolated brucellaphage lysed all the 12 *Brucella abortus* field isolates, *B. abortus* strain 99 and *Brucella melitensis* (procured from IVRI, Izatnagar) at MIC 1:50 but did not lyse any of the heterologous species tested viz. *Staphylococcus aureus, Salmonella species, Escherichia coli,* and *Pasteurella multocida*.

Morphological characterization of brucellaphage

Plaque morphology

The observed plaques were circular (Fig. 2) with a diameter of 0.5- 3.0 mm.

Phage morphology

The brucellaphage isolated in the present study was a tailed phage with icosahedral head (62.2 nm in diameter and 73.71 nm in length), and the head to tail length was 229.21 nm (Fig. 3).

Physicochemical characterization of the brucellaphage

Effect of temperature

The effect of temperature on the survivability of brucellaphage was studied. The phage titre gradually decreased from 4.5×10^6 to 2.0×10^6 pfu/ml within 48 h at 0^oC. Temperature treatments of -20^oC completely inactivated the phage within 4 h and treatment at 55^oC completely inactivated the phage within 1 hour (Table 1, Fig. 4).

Effect of pH on brucellaphage

Exposures to various pH (pH 2, 4, 6, 8, 10) were given to the brucellaphage to determine its survivability. The phage was inactivated at pH 2 within 4 h, and at pH 4 within 12 h. However the phage survived up to 24 h at pH 6.The phage number gradually decreased from 4.5×10^6 to 2.0×10^6 within 48 h at pH 8. It decreased to 0 within 24 h at pH 10 (Table 2, Fig. 5).

Effect of sunlight

When the brucellaphage was exposed to sunlight, the phage titre decreased gradually from 4.5×10^6 to 0.2×10^6 within 3 h and was completely inactivated after 4 h (Table 3, Fig. 6).

Effect of UV light

Exposure to UV light was found to have a drastic effect on phage survivability. The phage gets completely inactivated within 4 min. (Table 4, Fig. 7).

Chemical characterization of phage

Effect of chloroform (10%) on phage

The treatment of phage with 10% chloroform completely inactivated it within 15 min. at 37^oC (Table 5, Fig. 8).

Effect of SDS on phage

The effect of sodium dodecyl sulphate (SDS) treatment on activity of phage was studied. It was found that both 1% and 0.1% concentrations of SDS completely inactivated the phage within 15 min. at 37^oC (Table 6, Fig. 9).

Effect of enzymes on phage activity:

Effect of lysozyme

Lysozyme at a concentration of 20 mg/ml completely inactivated the phage within one hour (Table 7, Fig. 10).

Effect of RNase on phage

No detectable change was found on treatment of phage with RNase for 120 min. (Table 8, Fig. 11). According to Ackermann (2007) such types of phages belong to the order Caudovirales and family *Siphoviridae*.

We propose to name it as "Brucellaphage Ludhiana 1 (BpL1)". Cai-Zhong *et al.*, (2009) classified Tb (*Tbilisi*), as a member of the *Podoviridae* family with icosahedral capsids (57 ± 2 nm diameter) and short tails (32 ± 3 nm long). Chachra *et al.*, (2012) reported that electron microscopic studies of the brucellaphage revealed it to be an elementary body measuring approximately 65 nm with rounded head and a very short tail.

Table.1 Effect of temperature on survivability of brucellaphage

S. No	Time of incubation	0^0C	-20^0C	55^0C
1	0 min	4.5×10^6	4.5×10^6	4.5×10^6
2	30 min	4.0×10^6	1.5×10^6	0.3×10^6
3	1 hr.	3.6×10^6	0.2×10^6	
4	4 hr.	3.0×10^6		
5.	12 hr.	2.5×10^6		
6.	24 hr.	2.0×10^6		
7.	48hr.	2.0×10^6		

Table.2 Effect of pH on brucellaphage survivability

S. No	Time duration	pH 2	pH 4	pH 6	pH 8	pH 10
1	0 min	4.5×10^6	4.5×10^6	4.5×10^6	4.5×10^6	4.5×10^6
2	30 min	0.3×10^6	0.5×10^6	0.8×10^6	3.5×10^6	2.5×10^6
3	1 hr.	0.1×10^6	0.5×10^6	0.5×10^6	3.2×10^6	1.5×10^6
4	4 hr.		0.1×10^6	0.2×10^6	3.2×10^6	1.0×10^6
5.	12 hr.			0.1×10^6	2.5×10^6	0.2×10^6
6.	24 hr.			0.1×10^6	2.4×10^6	
7	48 hr.				2.0×10^6	

Table.3 Effect of sunlight on brucellaphage survival

S. No.	Duration of exposure	Phage count (Pfu/ml)
1	0 min	4.5×10^6
2	30 min	2.9×10^6
3	1 hr.	1.8×10^6
4	3 hr.	0.2×10^6
5	4 hr.	_
6	12 hr.	_

Table.4 Effect of UV light on brucellaphage

S. No.	Time duration of exposure	Phage count (Pfu/ml)
1	0 min	4.5×10^6
2	2 min	0.3×10^6
3	4 min	_

Table.5 Effect of chloroform on phage survival

S. No.	Time interval	Pfu/ml
1	0 min	3.9×10^6
2	10 min	0.2×10^3
3	15 min	_

Table.6 Effect of SDS treatment on phage

S. No	Time interval	Pfu/ml in 0.1% SDS	Pfu/ml in 1.0% SDS
1	0 min	4.4×10^6	4.2×10^6
2	10 min	0.3×10^6	0.1×10^6
3	15 min	_	_

Table.7 Effect of lysozyme on phage

S. No	Duration of exposure	Phage count (Pfu/ml)
1	0 min	3.5×10^6
2	10 min	1.0×10^6
3	30 min	0.1×10^6
4	60 min	_

Table.8 Effect of RNase on phage

S. No	Duration of exposure	Phage count (Pfu/ml)
1	0 min	4.4×10^6
2	10 min	4.2×10^6
3	30 min	3.9×10^6
4	60 min	3.5×10^6
5	120 min	2.5×10^6

Fig.1 Lytic zones along the streaking lines of BpL1 on Brucella lawn

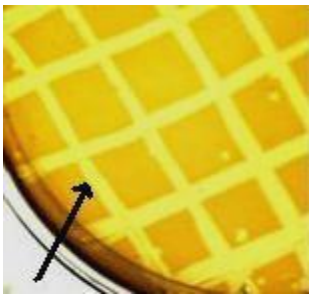


Fig.2 Circular plaques caused by BpL1 on *Brucella abortus* S19 lawn

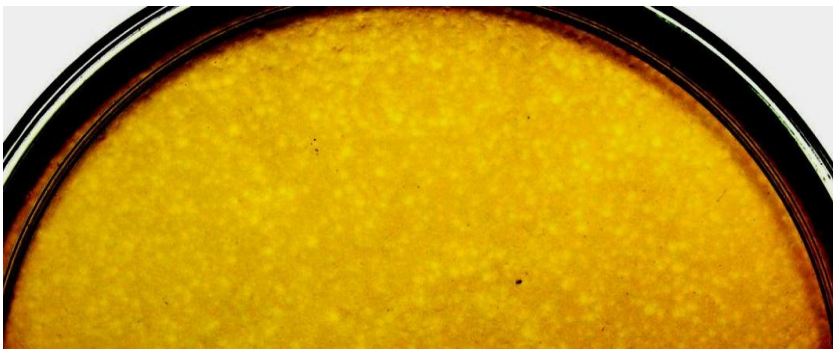


Fig.3 Electron micrograph of brucellaphage BpL1

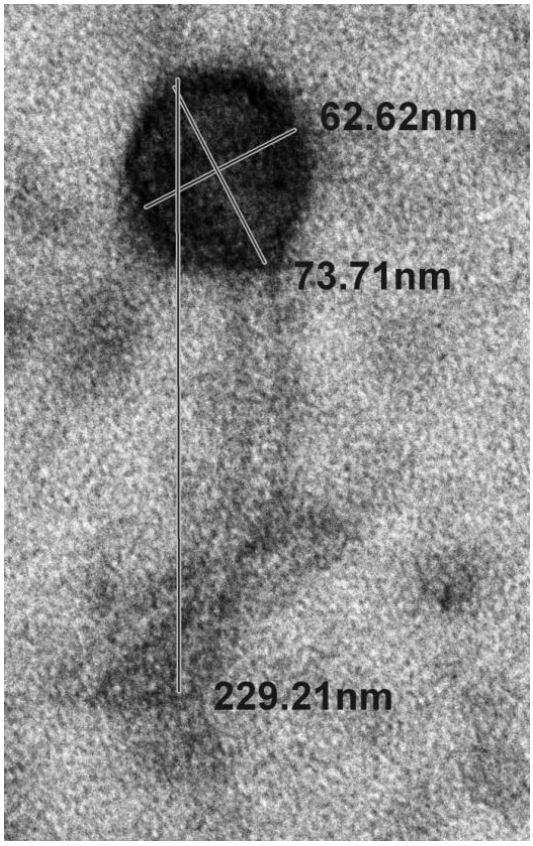


Fig.4 Effect of temperature on phage survival

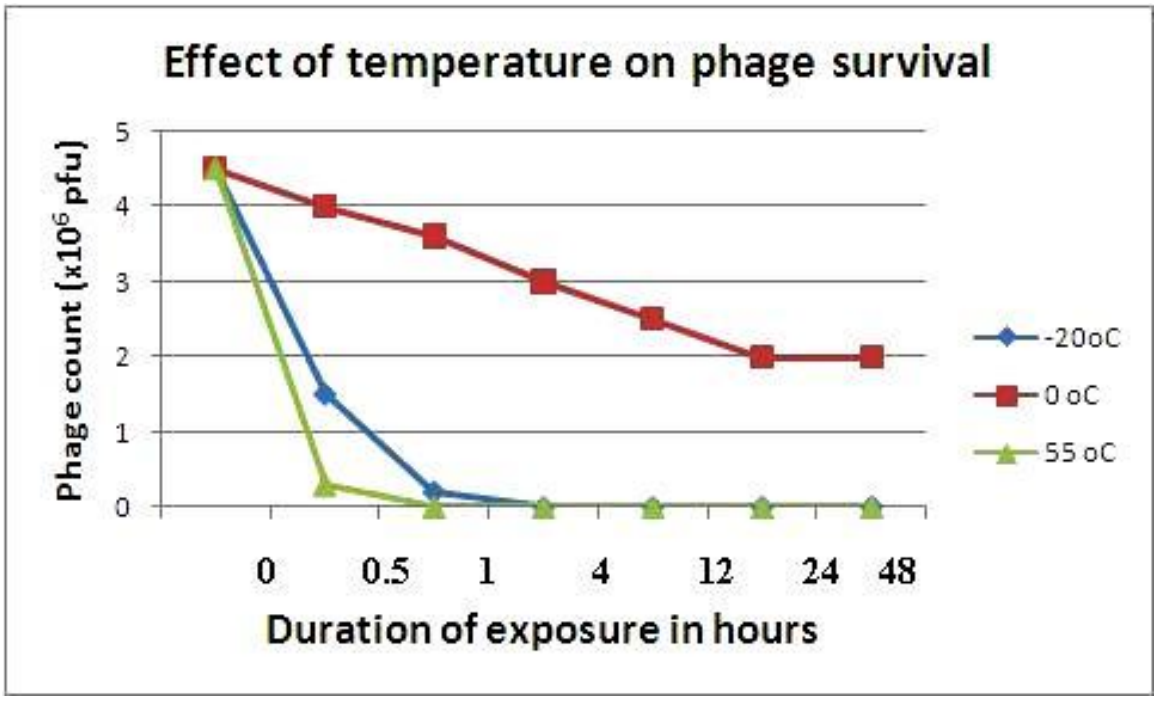


Fig.5 Effect of pH on phage survival

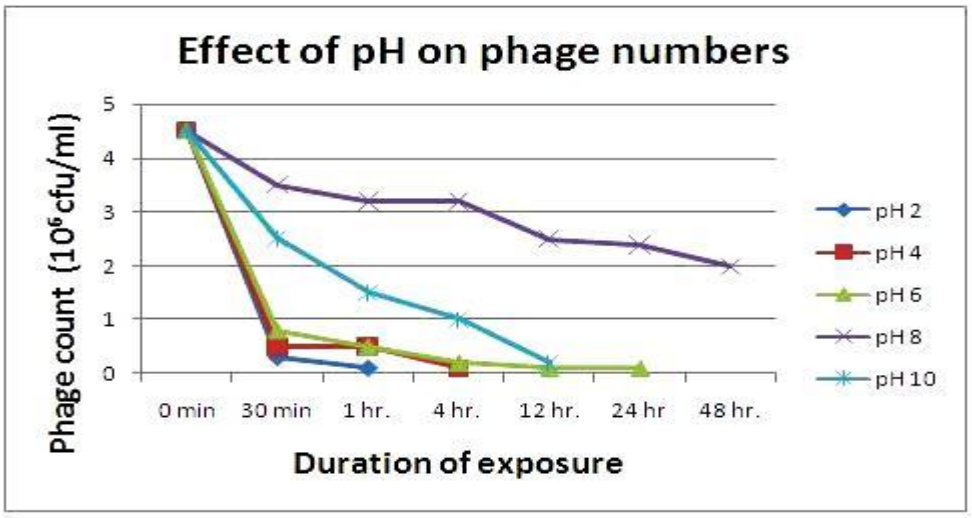


Fig.6 Effect of exposure to sunlight on brucellaphage survivability

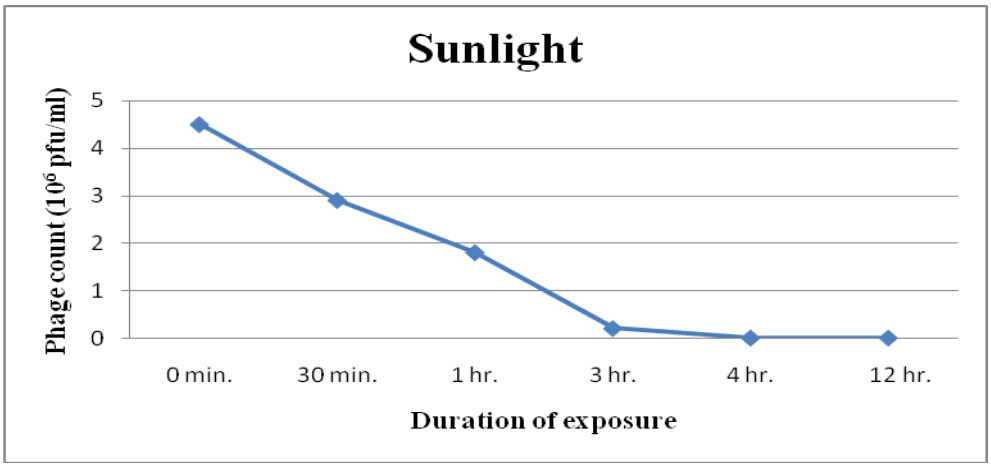


Fig.7 Effect of exposure to UV light on phage survival

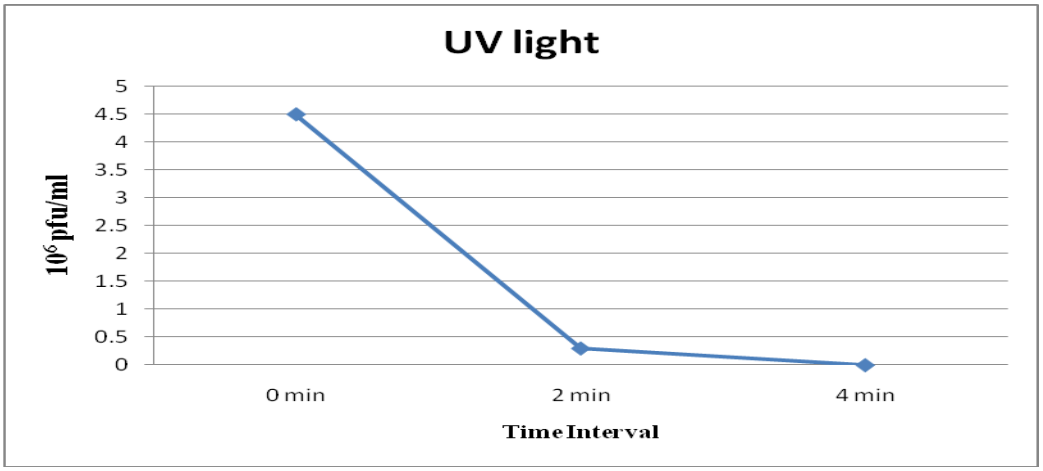


Fig.8 Effect of chloroform on phage survival

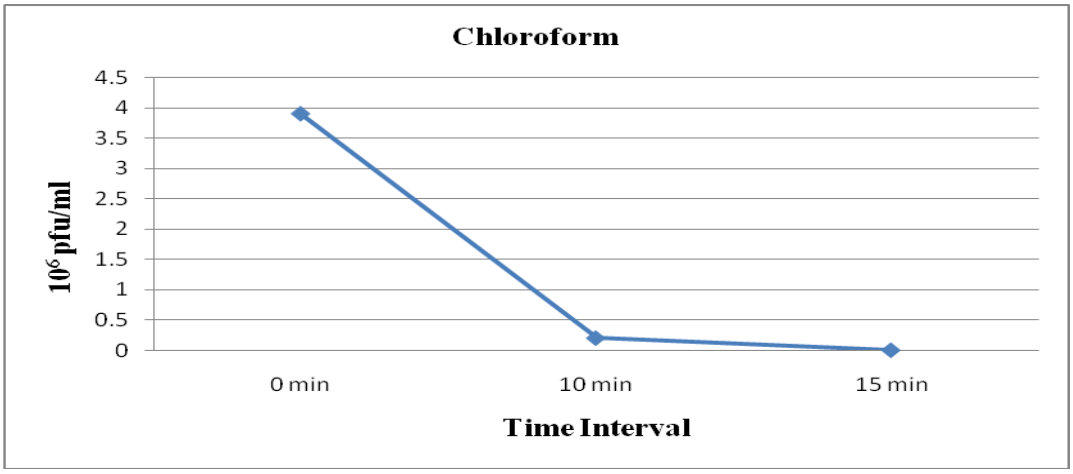


Fig.9 Effect of SDS on phage

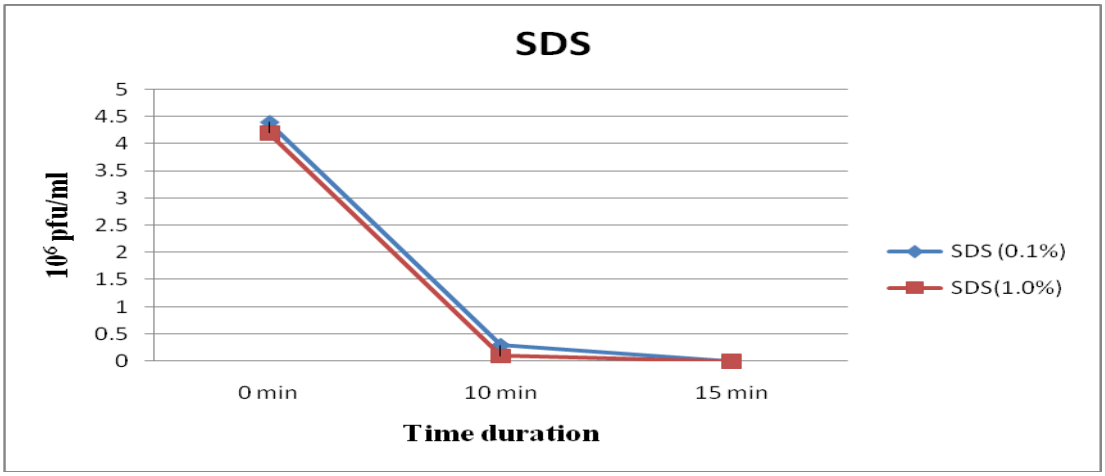


Fig.10 Effect of lysozyme on phage

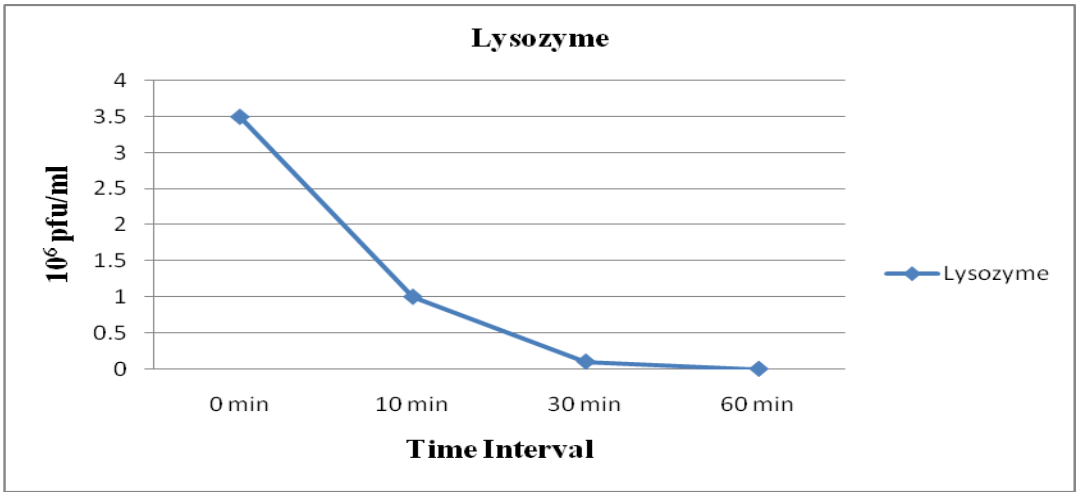
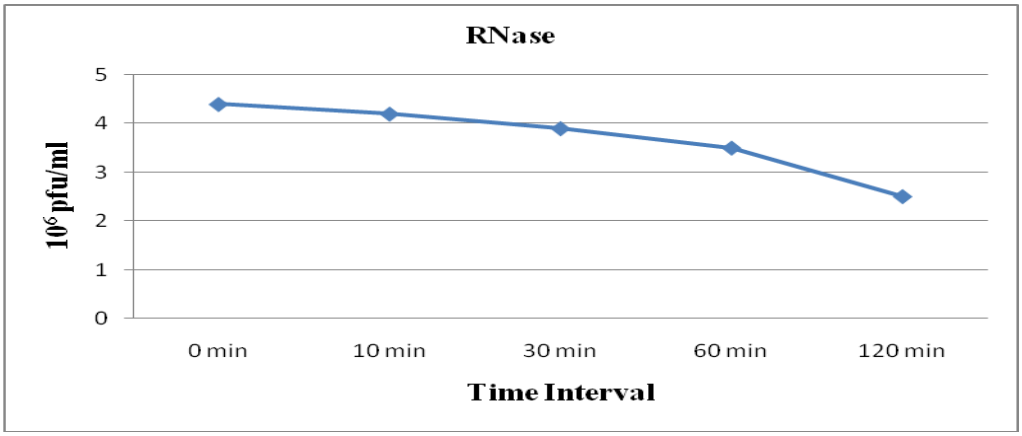


Fig.11 Effect of RNase on phage



Rigby *et al.,* (1989) reported that Nepean (Np) was morphologically identical to the other brucellaphages, with an icosahedral head (diameter 50-65 nm) and short tail (length 15-20 nm).

Our observations on lytic ability of phage were similar to those of Chachra *et al.,* (2012) and Pandey *et al.,* (2013) and who had reported that their brucellaphage did not lyse any of the heterologous bacterial species tested *viz. Staphylococcus aureus, Streptococcus* species, *E. coli, P. multocida, Pseudomonas* species and *Salmonella* Dublin. Morris and Corbel (1973) have reported that the isolated Weybridge phage was lytic for smooth *B. abortus* biotypes (1, 2, 3, 4, 5, 6, 7 and 9), *B. suis* biotypes (1, 2, 3 and 4), and *B. neotomae* cultures but there was no lysis of *B. canis, B. melitensis* and *B. ovis* strains. Pandey *et al.,* (2013) had reported clear plaques of brucellaphage of variable size (0.1 to 3 mm). Morris *et al.,* (1973) observed clear plaques of phage A422 of 0.1 to 2.0 mm diameter whereas the plaques of S708 and M51 were of two types i.e. small, turbid plaques, 0.1 to 0.5 mm in diameter, and large, clear plaques, 0.5 to 2.5 mm in diameter. McDuff *et al.,* (1961) had reported that 18% brucellaphages were inactivated at 60^{o}C and there was 100% inactivation at 70^{o}C within 60 min. Pandey *et al.,* (2013) have also reported that at 40^{o}C the brucellaphage titre gradually decreased from 1340 pfu/ml to110 pfu/ml within 3 h and treatment at 60^{o}C completely inactivated the phage within 10 min. Our observations were similar to the observations of McDuff *et al.,* (1961) who reported that there was no loss in phage titre in broth at pH values of 6.2 to 8.1 whereas, at pH 3.1 there was complete (100%) inactivation, 56% at pH 4.1, 24% at pH 5.0, 35% at pH 9.0 and 42% inactivation at pH 9.9, respectively. Pandey *et al.,* (2013) reported that treatment at pH 2 completely inactivated the phage within 3 h, whereas the phage titre gradually decreased to zero within 24 h at pH 4. At pH 6, there was only 38.9% decrease in the phage titre after 48 h of treatment. The phage remained stable at pH 8 with 75.31% survivability after 48 h treatment. At pH 10, the phage titre gradually decreased to 0.31% within 48 h. Pandey *et al.,* (2013) have also reported that direct sunlight gradually decreases the phage titre and within 3 h brucellaphage is reduced by 93.99% and exposure to UV rays inactivated the phage completely within 3 min.

McDuff *et al.,* (1961) had reported that there was 99% inactivation of phage by 10% chloroform within 5 min. Pandey *et al.,* (2013) had also reported the complete inactivation within 5 min. of exposure of phage to 10% chloroform, within 15 min. of SDS treatment and within 1 hour with lysozyme treatment. Morris *et al.,* (1973) had also reported no detectable effect on phage titre after RNase treatment for 90 min. Pandey *et al.,* (2013) had reported that phage remains almost stable up to 3 h after treatment with RNase.

Potential of phage for therapy

The new phage reported here is a broad acting lytic brucellaphage and can be evaluated in vivo for effective therapy of bovine brucellosis.

We have isolated a broad acting lytic brucellaphage. It was a tailed phage with icosahedral head. The phage belonged to the order Caudovirales and family *Siphoviridae*.

Conflict of interest

The authors declare that there is no conflict of interest.

References

Ackermann H W. 2007. 5500 Phages examined in the electron microscope. *Arch. Virol.* 152: 277-43.

Adams M H. 1959. Bacteriophages. Interscience, New York.

Cai-Zhong Z, Hong-Yan X, Jing H, Bu-Yun C, Dong-Ri P, Ya-Fei L, Hai J, Qian R, Xiang-Yu M, Ya-Ming C, Xia H, Hong-Yan Z and Lan-Yu L. 2009. Molecular characterization of Tb, a new approach for an ancient brucellaphage. *Int. J. Mol. Sci.* 10: 2999-11.

Calderone J G and Pickett M J. 1965. Characterization of Brucellaphages. *J. Gen. Microbiol.*, 39, 1-10.

Chachra D, Kaur H, Chandra M and Saxena H M. 2012. Isolation, electron microscopy and physicochemical characterization of a brucellaphage against *B. abortus* Vaccine Strain S19. *Internet J. Microbiol.* 10(2).

Chilamban C, Rawat M and Somvanshi R. 2004. Pre-clinical studies on therapy of *S. aureus* mastitis by bacteriophage in mice model. *Ind. J Comp. Microbiol. Immunol. Infect. Dis*. 25: 98-103.

Gupta, V and Saxena H M (2017) Isolation, biochemical characterization and PCR confirmation of antibiotic resistant *Brucella abortus* from cattle and buffaloes in Punjab state of India. Res. Environ. Life Sci. 10(8) 718-723.

Mathur B G, Amarnath S K and Shinde R S. 2007. Review of clinical and laboratory features of human brucellosis. *Ind. J. Med. Microbiol.* 25: 188-02.

McDuff C R, Jones L M and Wilson J B. 1961.Characteristics of brucellaphages. *J. Bacteriol.* 83: 324-29.

Mohan A, Saxena H M, Malhotra P (2016) A comparison of titers of anti-*Brucella* antibodies of naturally infected and healthy vaccinated cattle by standard tube agglutination test, microtiter plate agglutination test, indirect hemagglutination assay, and indirect enzyme-linked immune sorbent assay. *Vet. World 9*(7): 717-722

Morris J A and Corbel M J. 1973. Properties of a new phage lytic for *Brucella suis*. *J. Gen. Virol.* 21: 539-54.

Morris J A, Corbel M J and Phillip J I H. 1973.Characterization of three phages lytic for *Brucella* species. *J. Gen. Virol.* 20: 63-73.

Pandey S, Chachra D, Chandra M and Saxena H M. 2013.Protein profiling and physico-chemical characterization of an isolated phage against *Brucella abortus* strain 19. *Afr. J. Microbiol. Res.* 7(14):1233-38.

Rigby C E, Cerqueira-Campos M L, Kelly H A and Surujballi O P. 1989. Properties and partial genetic characterization of Nepean Phage and other lytic phages of *Brucella* Species. *Canad. J. Vet. Res.* 53(3): 319-25.

Romero C, Gamazo C, Pardo M and Lopez-Goni I. 1995. Specific detection of *Brucella* DNA by PCR. *J. Clin. Microbiol.* 33(3): 615-17.

Sambrook, J. and Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual; Coldspring Harbor Laboratory Press NewYork, USA.

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