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Full Length Research Paper

Protein profiling and physico-chemical characterization of an isolated phage against *Brucella abortus* strain 19

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Bacteriophages have varied practical applications, so we did a basic study of the characteristics of an isolated phage against *Brucella abortus* for proper understanding of its general properties. Bacteriophage against *B. abortus* strain 19 was isolated from the sewage samples. Structural protein profiling of the isolated brucellaphage by SDS-PAGE revealed 4 bands of molecular weight: 65.98, 60.46, 48.56 and 43.97 kDa. The brucellaphage was susceptible to heat at and above 60°C temperature. Exposure to sunlight gradually decreased the phage concentration within 3 h. Ultraviolent light (UV) completely inactivated phage within 3 min of exposure. The brucellaphage was susceptible to pH 2, 4 and 10, whereas pH 6 and 8 did not affect the phage activity. The phage was sensitive to SDS, phenol, chloroform, iodine, benzalkonium and formalin, whereas it was insensitive to EDTA treatment. Lysozyme completely inactivated the phage within one hour, while only 0.51% phage survival was observed in the presence of trypsin within 3 h, whereas RNase treatment did not affect the phage titre. The phage isolated was *Brucella* specific and did not lyse any of the heterologous bacterial species tested. The phage lysed *B. abortus* vaccine strains S19 and S99; *Brucella melitensis* and *B. abortus* field isolates.

Key words: Brucellaphage, *Brucella abortus* strain 19, physicochemical characterization.

INTRODUCTION

Brucellosis is an economically important disease of public health; it is caused by members of genus *Brucella*. It is endemic in India and is prevalent in all the states of the country including Punjab (Aulakh et al., 2008). The main economic impact of infection on animals is because of the reproductive failure and losses incurred due to the death of foetuses. Based on the epidemiological data of active surveillance programme, it is estimated that due to Brucellosis, there is a loss of US$58.8 million per year in India (Kollannur et al., 2007).

The bacteriophages that infect *Brucella* species are called brucellaphages. All the brucellaphages that have been described to date belong to the order *Caudovirales*, family *Podoviridae* (Ackermann, 2007). These are icosahedral phages with short tails and linear double-stranded DNA. On the basis of host range, six brucellaphage groups have been identified, that is Tbilisi (Tb), Weybridge (Wb), Firenze (Fi), R, Izatnagar (Iz) and Berkeley (BK) (WHO, 1986). The brucellaphages that have been described differ mainly in the species of *Brucella* which they infect and their activity is highly sensitive to smooth-rough variation in their host

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brucellae, that is,Tb and Fi phages replicate in smooth *Brucella abortus* (Rigby et al., 1989), Wb replicates in smooth strains of both *Brucella suis* and *B. abortus* and *Brucella neotomae* (Morris and Corbel, 1973), Iz replicates in smooth strains *B. melitensis, B. suis, B. abortus,* rough strains of *Brucella melitensis* and *B. suis* and, to a lesser extent *Brucella ovis* (Corbel et al., 1988). These lytic activity patterns are important in diagnosis and therapy of the disease caused by a particular species.

In the present study, we analysed the protein profile, physicochemical characteristics, enzymatic resistance and host range of the brucellaphage isolated from sewage samples from the dairy farms in and around Ludhiana, India. Isolation and characterization of brucellaphage is important since it will facilitate their wider use in various practical applications of diagnosis and therapy.

MATERIALS AND METHODS

Bacterial strain used

B. abortus strain S 19 (isolated from live *Brucella* S 19 vaccine procured from Punjab Veterinary Vaccine Institute, Ludhiana, India) was used for isolation and propagation of brucellaphage.

Isolation of brucellaphage

Sewage samples were collected in a wide mouth bottle from dairy farms in and around Ludhiana, India. Briefly, 10 ml of the 24 h culture of *B. abortus* strain S 19 in *Brucella* broth (Himedia, Mumbai) was put into 25 ml of double strength NZCYM broth (Amresco) and then 15 ml of the sewage sample was poured into it and was incubated at 37°C. This processed sample was then filtered through 0.22 µm PVDF filter (Axiva) and inoculated by agar overlay technique (McDuff et al., 1961) on alternate days (viz. 4, 6, 8 up to 20 days) for bacteriophage isolation. Plates having plaque formation (clear lysis, appreciable by naked eyes, on *Brucella* lawn) were preserved and the plaques were confirmed for phage by secondary streaking.

Propagation of brucellaphage

The phage confirmed by secondary streaking was eluted from the plates into SM buffer and was passed through 0.22 µm PVDF filter. It was then preserved at 4°C till further use. The eluted phage was concentrated at 200000 ×g at 4°C for 4 h in ultracentrifuge (Hitachi, Japan). The supernatant was discarded and the pellet was re-dissolved in 0.5 ml of sterile SM buffer and stored at 4°C till further use.

Estimation of the titre of the brucellaphage

The purified brucellaphage was serially diluted in SM buffer. Equal volume of each phage dilution and fresh *Brucella* (S 19 strain) broth culture were mixed in 5 ml of semisolid NZCYM agar and then poured on preformed NZCYM + BSM (*Brucella* Selective Medium) agar plates. The plaques produced were counted and the titre was expressed in plaque forming units (pfu)/ml (dilution factor was taken into account).

Protein profile

The structural proteins of brucellaphage were observed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The ultracentrifuged purified phage preparation was concentrated in a vacuum evaporator (Eppendorf Concentrator 5301) to one-fourth of the volume and then later subjected to SDS-PAGE as per the method of Laemmli (1970). The concentrated samples were mixed with 5x sample buffer and boiled at100°C in a water bath for 10 min. Standard pre-stained protein molecular weight marker with a range of 16 to 100 kDa (MBI, Fermentas) was used to estimate the molecular weights. The gel was visualised using Gel Doc system (AlphaInnotech).

Physical characterization

The effect of heat, sunlight, UV light and pH on survivability of phage was determined. To determine the heat stability, purified phage preparation was subjected to the test temperature (40 and 60°C) in a water bath. After periodic time intervals, that is, 5, 10, 30, 60, 120 and 180 min, the aliquots (100 µl) were used to calculate phage titre (pfu/ml) in order to estimate the survivability relationship. Similarly, the effect of direct sunlight on purified phage preparation was determined. After various time intervals, that is, 15, 30, 60, 120 and 180 min, the aliquots (100 µl) were used for estimation of survivability in terms of phage titre. The brucellaphage was then treated with UV light for the same cause. For this, purified phage preparation was treated with UV light for 1, 3 and 5 min. Then, the aliquots (100 µl) were used for estimation of phage titre. The effect of various pH conditions, that is, 2, 4, 6, 8 and 10 on the phage survivability was also observed. The purified phage preparation was mixed with TE buffer having the test pH in 1:10 ratio and incubated at 37°C. At various time intervals, that is, from 30 min to 48 h, the aliquots (1 ml of each) were used for estimation of survivability of phage in terms of phage titre.

Chemical characterization

Effect of some commonly available chemicals, that is, SDS (0.1 and 1%), EDTA (0.01 M), phenol (5% aqueous), chloroform (10%), iodides (5% iodine and 10% potassium iodide in water), benzalkonium chloride (0.5%) and formalin (40%) on survivability of brucellaphage was also observed. The purified phage preparation was mixed with an equal volume of the test chemical and incubated at 37°C. After regular time intervals, the aliquots were examined and were subjected to estimate phage titre.

Effect of enzymes

The effect of trypsin (1.25 mg/ml), lysozyme (20 mg/ml) and RNase (10 mg/ml) on survivability of phage was observed. The purified phage preparation was mixed with equal volume of the test enzyme and incubated at 37°C. After periodic time intervals (up to 180 min), the aliquots were taken out to estimate phage titre.

Determination of host range

The lytic activity of brucellaphage was assessed against nine *B. abortus* field isolates (isolated as M.V.Sc. thesis work by Pandey, 2011), *B. abortus* strain 99 (procured from Punjab Veterinary Vaccine Institute, Ludhiana, India) and *B. melitensis* (procured from

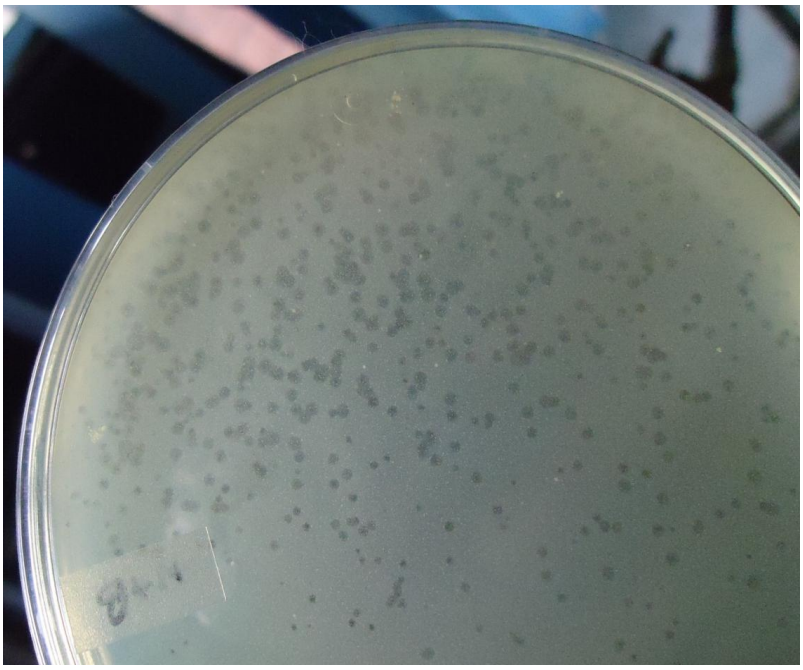

Figure 1. Plaques morphology; circular plaques with diameter ranging from 0.1 to 3 mm.

IVRI, Izatnagar, India). Heterologous species viz. *Staphylococcus aureus, Streptococcus* species, *Escherichia coli, Pasteurella multocida, Pseudomonas* species and *Salmonella* Dublin (isolated from field cases presented in the Department of Veterinary Microbiology, GADVASU, Ludhiana) were also tested to determine the host range of brucellaphage.

In brief, 200 µl of the overnight broth culture of the test organism (as indicated above) was individually mixed with 5 ml of semisolid NZCYM agar. It was then poured on NZCYM agar plates and allowed to solidify at room temperature. The isolated phage was then streaked on these solidified plates and incubated at 37°C under aerobic condition. Each plate was observed for 2 to 3 days for any clearing along the phage streak lines.

RESULTS AND DISCUSSION

A total of 56 sewage samples were collected and processed for isolation of brucellaphage. The plaques were observed on day 5 to 6 of primary isolation. The observed plaques were circular with a diameter of 0.1 to 3 mm (Figure 1).

Physical characterization

The effect of heat on the activity of phage revealed that at 40°C phage titre gradually decreased from 1340 to 110 pfu/ml (8.2%) within three hours. 60°C temperature treatment completely inactivated the phage within 10 min (Figure 2).

The effect of sunlight on activity of phage revealed that exposure to direct sunlight gradually decreases the phage concentration and within 3 h, brucellaphage titre gets reduced by 93.99%. The effect of UV light on activity of phage revealed that UV light has drastic effect on the phage survivability. The phage gets completely inactivated within 3 min (Figure 3).

The brucellaphage when subjected to various pH range viz. pH 2, 4, 6, 8 and 10 indicated that the pH 2 treatment completely inactivated the phage within 3 h, whereas the phage titre gradually decreased to zero within 24 h at pH 4 and at pH 6, there was only 38.9% decrease in the phage titre within 48 h of treatment. The phage remained stable at pH 8 with 75.31% survivability in 48 h. At pH 10, phage titre gradually decreased to 0.31% within 48 h (Figure 4). These observations were similar to the observations of McDuff et al. (1961) who observed that there was no loss in phage titre in broth at pH values of 6.2 to 8.1 whereas, there was complete inactivation (100%) at pH 3.1, 56% at 4.1, 24% at 5.0, 35% at 9.0 and 42% at 9.9.

Chemical characterization

SDS, phenol, iodine formalin and benzalkonium completely inactivated the phage within 15 min at 37°C whereas with chloroform, complete inactivation was observed within 5 min. Similar observation was reported by McDuff et al. (1961) who observed 99% inactivation of the brucellaphage on treatment with 10% chloroform for 5 min.

The brucellaphage remained stable in 0.01 M EDTA

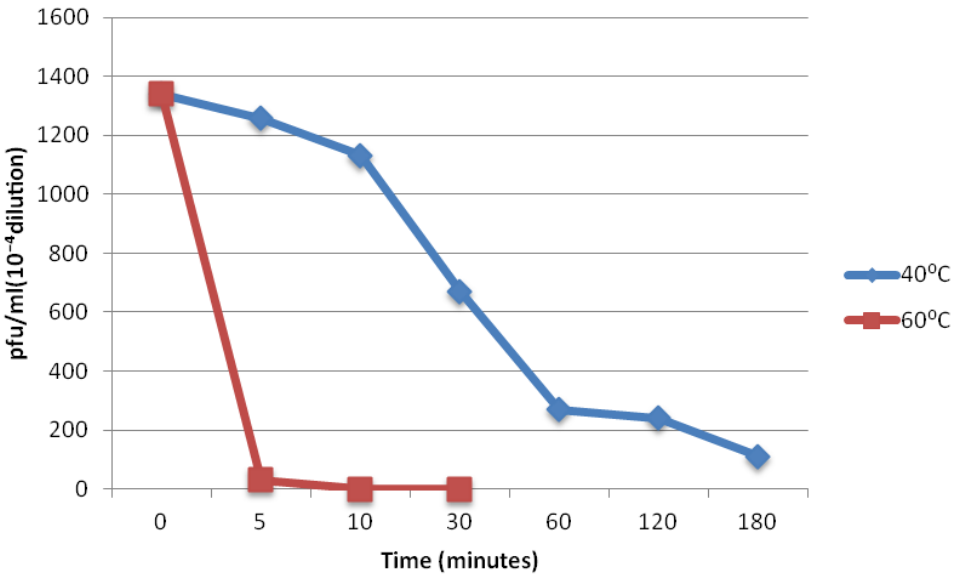

Figure 2. Effect of heat on phage survivability.

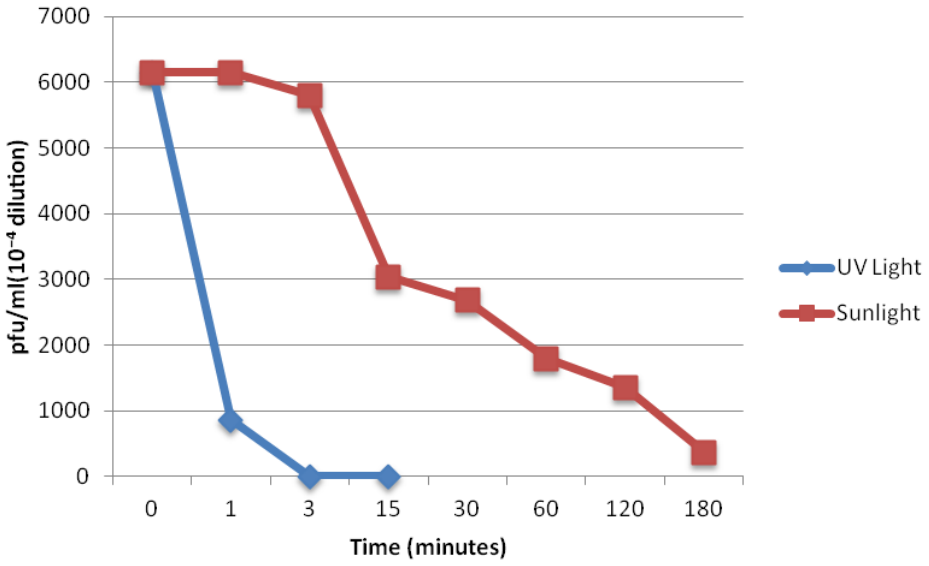

Figure 3. Effect of sunlight and UV light on phage.

solution for 3 h with 95.65% survivability. Similarly, Morris and Corbel (1973) observed 98% survivability of Tbilisi phage in 0.01 M EDTA in 90 min.

Effect of enzymes

Within 3 h, only 0.51% phage survived the treatment with the proteolytic enzyme trypsin at 37°C. Lysozyme completely inactivated the phage within one hour. The phage remained stable on RNase treatment for three hours (Figure 5). Similarly, Morris et al. (1973) reported only 10% survivability of Tbilisi phage on treatment with trypsin at 10 mg/ml concentration for 90 min, they also reported no detectable effect on phage titre on RNase treatment for 90 min.

Proteomics

On SDS-PAGE, four bands of structural proteins were obtained with a molecular weight of 65.98, 60.46, 48.56

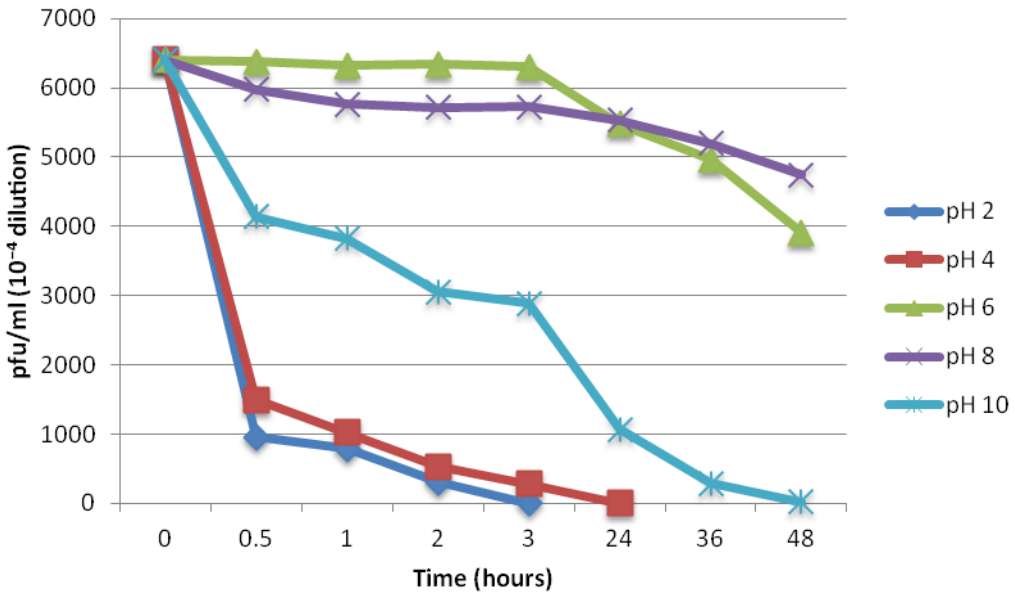

Figure 4. Effect of pH on phage survivability.

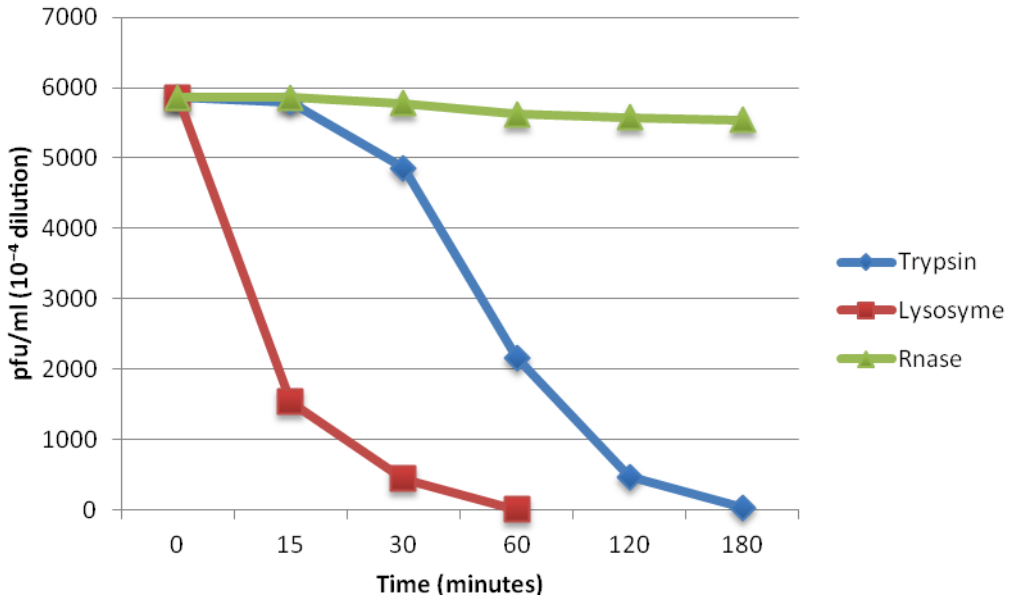

Figure 5. Effect of enzymes on phage survivability. Trypsin (1.25 mg/ml), lysozyme (20 mg/ml) and RNase (10 mg/ml).

and 43.97 kDa (Figure 6). Similarly, Zhu et al. (2009) observed nine bands, ranging from 40 to 85 kDa of the structural proteins of Tbilisi phage.

Host range

The phages isolated in the study lysed all the nine *B. abortus* field isolates, *B. abortus* strain 99 and *B. melitensis*. The phages did not lyse any of the heterologous bacterial species tested *viz*. *S. aureus, Streptococcus* species, *E. coli, P. multocida, Pseudomonas* species and *Salmonella* Dublin. The above observations were similar to Calderone and Pickett (1965) who reported that the brucellaphage did not lyse any of the heterologous bacterial species tested (*Pasteurella tularensis, Pasteurella novicida, Pasteurella multocida, Pasteurella haemolytica, Pasteurella*

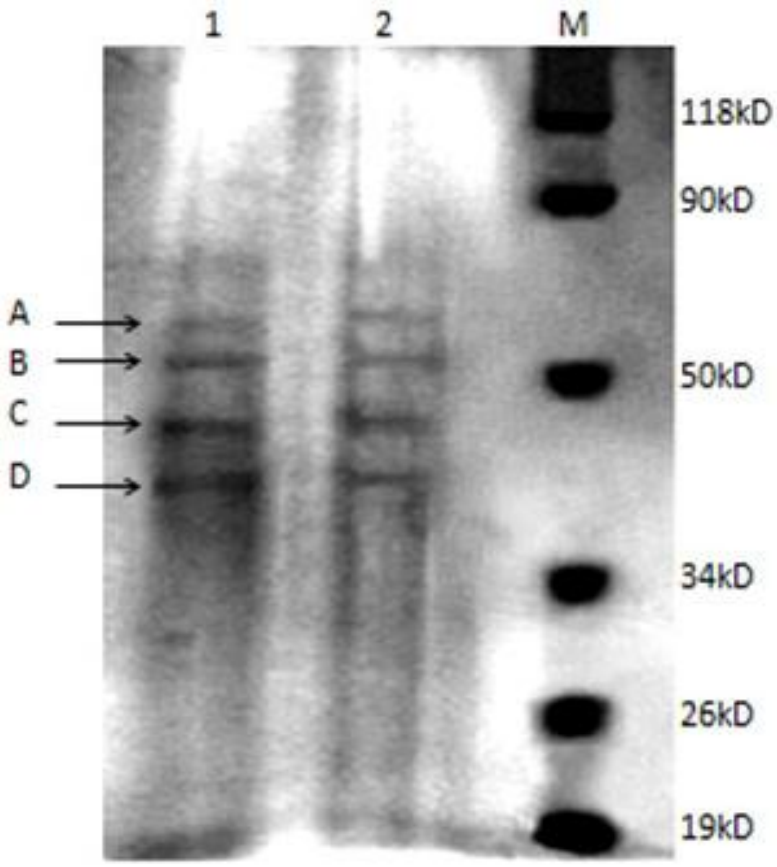

Figure 6. SDS-PAGE image showing bands of brucellaphage proteins of molecular weights; A: 65.98 kDa, B: 60.46 kDa, C: 48.56 kDa and D: 43.97 kDa; M: protein marker; Lanes 1and 2: brucellaphage sample in duplicate.

pseudotuberculosis, Pasteurella pestis, Haemophillus influenzae, Bordetella parapertusis, Bordetella bronchiseptica and *Bordetella pertusis*).

Conclusions

In this study, we isolated a bacteriophage against *B. abortus* S-19 and characterized it on the basis of physicochemical characteristics and protein profiling which will pave the way for its further use as therapeutic and diagnostic tool.

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