# Isolation, Electron Microscopy And Physicochemical Characterization Of A Brucellaphage Against Brucella Abortus Vaccine Strain S19

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**Citation**

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**Abstract**

Brucellosis is an economically important but neglected emerging endemic zoonotic communicable disease in India. The prevalence of brucellosis has increased from 34.15% of the samples in 2006-07 to 67.28 % in the year 2010-11. Brucella abortus is an intracellular pathogen capable of infecting animals and humans. The use of phages in the treatment of bacterial infections is an attractive alternative to existing antibiotic therapy. Phages target a particular host and it is unlikely to elicit resistance in untargeted bacterial strains. A brucellaphage was isolated against actively growing stage of Brucella abortus strain S 19 from sewage sample of a dairy farm in Ludhiana. The plaque morphology revealed discrete, clear, circular plaques of diameter 0.1 to 3 mm after 48 h of incubation at 37°C aerobically. The field isolates (n=9) of B. abortus were sensitive to phage. The host range of brucellaphage is against vaccine strains, viz. S19, S99 and Rev1 of B. abortus, B. melitensis and B. suis. The isolated brucellaphage failed to lyse any culture of heterologous species tested viz. Pasteurella multocida, E.coli, Staphylococcus, Streptococcus, Salmonella Dublin, Micrococcus and Pseudomonas spp. Electron microscopic studies of the brucellaphage revealed it to be an elementary body measuring approximately 65 nm at 50,000 X magnification with rounded head and a very short tail.The size and shape resembles another Brucella phage Tbilisi phage and the other phages isolated elsewhere. The isolated brucellaphage was able to survive at a temperature of -20°C, 4°C, 37°C and 50°C when exposed for duration of 20 min. But, a temperature of 70°C and beyond was lethal for the brucellaphage. Unlike normal light, the effect of sunlight on the survivability of phage indicates deleterious effects on the phage. UV light completely destroyed the phage within 15 min. Non-ionic detergents like SDS (10%) completely destroyed the phage in 15 min. There was no effect of RNAse and trypsin on the survivability of phage while proteinase K and lysozyme reduced the survivability of the isolated phage. The isolated phage was tolerant to pH 7 and 9 while there was a reduction in phage activity at pH 3 and 5. According to the literature reviewed, this is the first report of isolation of a genus specific brucellaphage against B. abortus from Punjab which will pave a way for its use in various cost effective diagnostics and in therapy of brucellosis.

## INTRODUCTION

Brucellosis is an economically important but neglected emerging endemic zoonotic communicable disease in India. The prevalence of brucellosis has increased from 34.15% of the samples in 2006-07 to 67.28 % in the year 2010-11. Brucellosis is a zoonotic disease of worldwide importance caused by Brucella abortus. It is characterized by abortions, stillbirths and reproductive problems in animals (Garin Bastuji et al 2008). The disease is associated with domestic and wild animals and human beings get infected accidently by contact with vaginal discharges, fetal fluids (Garin Bastuji et al 2008) or by ingestion of unpasteurized milk (Pappas et al. 2005). Among 8 species of Brucella, 3 are known to be virulent for human beings (B. melitensis, B. abortus and B. suis (Fugier et al. 2007). Viruses are obligate intracellular parasites that require specific host cell for its replication (Mayer, 2005). Bacteriophages are viruses that exclusively target and reproduce within bacterial cells. Generally they use their tail fibres to attach to their host cell and inject their nucleic acid into the bacterium. The host cell machinery is then used by the phage to replicate and get incorporated into the protein capsid. The mature viruses are released on the lysis of cell membrane of bacterium. The property of specificity of viruses makes them effective in fighting specific bacterial infections. Their use in waste water treatment has been well documented by Sulakvelidze and Burrow, 2005; Sulakvelidze and Kutter, 2005 and Withey et al. 2005. The use of phages in the treatment of

bacterial infections has come up as an alternative to existing antibiotic therapy. Unlike the broad spectrum antibiotic therapy, phages target a particular host and unlikely elicit resistance in untargeted bacterial strains.

Existence of bacteriophages against Brucella has been reported by researches long time back. Since then brucellaphages have been used in speciation and biotyping of brucellae, but little is known of the brucellaphage-host cell relationship. Molecular characterization of reference phage Tbilisi phage DNA has recently been reported by Zhu et al. 2009. The objectives of this study were to isolate, identify and characterize Brucella abortus from foetal stomach contents, vaginal and cervical discharges from cattle and buffaloes with a history of abortions and repeat breeding and secondly to isolate brucellaphage against B. abortus vaccine strain S 19 from sewage samples and to characterize it on the basis of its plaque morphology, electron microscopic features and lysis pattern of other Brucella species and a few heterologous species of bacteria.

## MATERIALS AND METHODS

### ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF

Brucella abortus vaccine strain S19, Brucella abortus S99, B. melitensis Rev1 and B. melitensis were taken from Indian Veterinary Research Institute (IVRI), Izatnagar. Bacteria were grown on Brucella selective agar (HiMedia) incubated at 37°C aerobically. B. abortus from field isolates (n=9) were isolated on Brucella selective agar. A total of 76 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 from different farms in and around Ludhiana, Punjab 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 were done by Polymerase chain reaction (PCR) as per the method of Romero et al. 1995

### ISOLATION OF BACTERIOPHAGE AGAINST

Agar overlay technique was used to isolate bacteriophage against Brucella abortus (Adams 1959 and Chilamban 2004). A total of seven sewage samples were collected from nearby dairy farms 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°C. Out of this incubated sewage + bacteria cocktail 10 ml of supernatant was taken every day and centrifuged at 8000g for 15 minutes to collect the supernatant which was passed through 0.22µm PVDF filter (Axiva) and the filtrate was aseptically collected and stored at 4°C till further use and was designated as Bacteria Free Filtrate (BFF). Equal quantities (100 µl) of BFF and o / n broth culture of B. abortus were mixed in 0.75% NZCYM agar (maintained at 45°C in a dry bath) and was spread evenly over 1.5% NZCYM agar + BSM agar. The soft agar was allowed to solidify and the plates were incubated at 37°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°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 pieces and then the supernatant was filtered through 0.22 µm filters to remove the bacteria and elute the phage in SM buffer.

### HETEROLOGOUS SPECIES OF BACTERIA

A set of heterologous species of bacteria of veterinary importance viz. Pasteurella multocida B: 2, E.coli, Staphylococcus, Streptococcus, Salmonella Dublin, Micrococcus and Pseudomonas spp were available in the department of Veterinary Microbiology. These bacteria were identified as per the standard protocols of Quinn and Carter (1994).

### TRANSMISSION ELECTRON MICROSCOPY

Transmission Electron Microscopy (TEM) was done using Hitachi Model H-7650 with 40-120 KV accelerating voltage; 0.2 nm resolutions and 1024 X 1024 pixels digital camera at Electron Microscopy and Nanoscience Laboratory, Punjab Agricultural University, Ludhiana

### EFFECT OF VARIED TEMPERATURES ON THE BRUCELLAPHAGE

100µl of brucellaphage ($10^6$pfu/ml) was subjected to

temperatures of -20°C, 4°C, 37°C, 50°C, 70°C and 100°C for a period of 20 min. 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 LIGHT ON THE BRUCELLAPHAGE

100µl of brucellaphage ($10^6$pfu/ml) was subjected to normal fluorescent tube light, sunlight and UV light for a period of 15 min to 90 min. 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 ENZYMES ON THE BRUCELLAPHAGE

100µl of brucellaphage ($10^6$pfu/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. 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 SDS (10%), NSS, EDTA (0.01M) ON THE BRUCELLAPHAGE

100µl of brucellaphage ($10^6$pfu/ml) was subjected to treatment with equal volume of SDS(10%), 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 ($10^6$pfu/ml) was observed for the change in pfu count in different pH ranges of 3, 5, 7 and 9 for a period of 30 min exposure time 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.

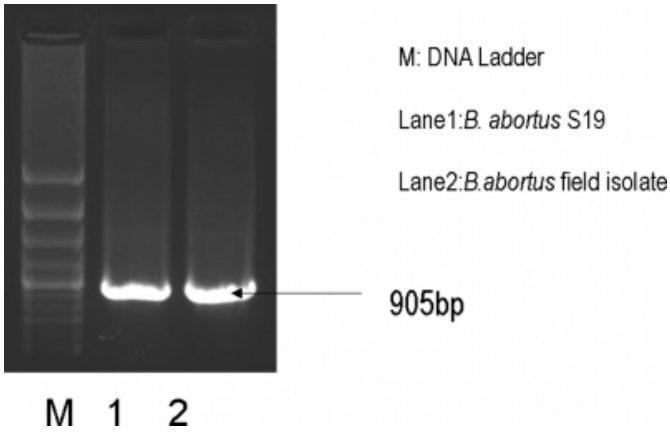
## RESULTS AND DISCUSSION

## ISOLATION OF

B. abortus was isolated from nine samples (all aborted foetal stomach contents) out of a total of 76 samples comprising of aborted foetal stomach contents, placenta and cotyledons and vaginal and uterine secretions from cattle and buffaloes with a history of abortions. The appearance of pin point, smooth, glistening and translucent colonies after 3-5 days of incubation on Brucella selective medium at 37°C in a microaerophillic environment were indicative of Brucella. Gram negative coccobacillary rods were observed in Gram's staining and Modified Ziehl Neelson staining red coccobacilli with a blue background. All the nine field isolates were positive for oxidase, catalase, urease and nitrate reduction, and negative for indole production. All these isolates were agglutinated by standard Brucella abortus antiserum. On the basis of dye sensitivity test all the isolates belonged to biotype I as all were growing in the presence of basic fuchsin (20µg/ml) and not in the presence of thionine dye (20µg/ml). All the field isolates (n=9) were confirmed by PCR based upon the amplification of band size of 903 bp as observed under gel electrophoresis (Fig.1).

**Figure 1**

Fig 1 Gel Electophoresis of S19 and field isolate of



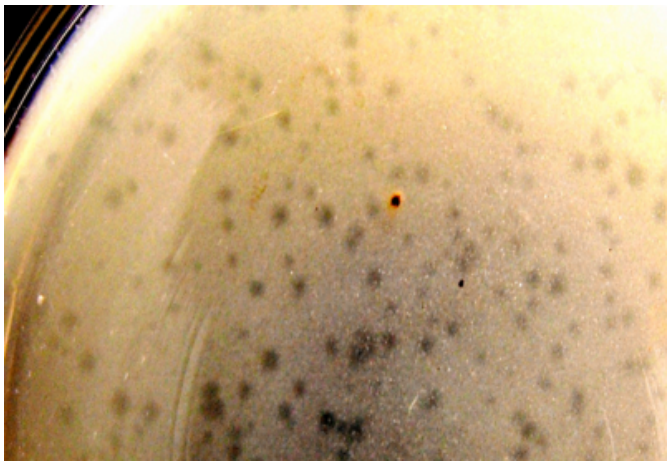

## ISOLATION OF BRUCELLAPHAGE

A brucellaphage was isolated against actively growing stage of Brucella abortus strain S19 from a sewage sample of a dairy farm in Ludhiana on day 6 $^{th}$ of incubation. Isolation of a brucellaphage from sewage sample from a dairy herd reveals the presence of phages in an environment. Phages are abundant in natural environment. They exceed bacteria by atleast one order of magnitude (Chibani-Chennoufi et al. 2004).The plaque morphology revealed discrete, clear, circular plaques of diameter 0.1 to 3 mm (Fig.2) after 48 h of incubation at 37°C aerobically. The plaque morphology

resembled those reported by Mc Duff et al. 1961. The maximum size of the plaques was obtained after 48 h of incubation and their size ranged from 0.5 to 5 mm in diameter. The isolation of phages was tried from the first day of incubation of sewage sample and indicator strain in NZCYM 2x broth. However, no isolation of phages for the first 6 days of incubation could be because of the long generation time of the organism i.e approximately 4h in broth as also reported by McDuff et al. 1961. Tbilisi is a typical brucellaphage and is an international reference strain in reference to its morphology, host range and resistance to chemical and physical agents (Zhu et al. 2009). The results of our brucellaphage are in general agreement with those of previous studies (Ackermann et al 1981, Corbel and Morris, 1980,).

**Figure 2**

Fig.2 Plaques of brucellaphage



## LYTIC ACTIVITY OF BRUCELLAPHAGE

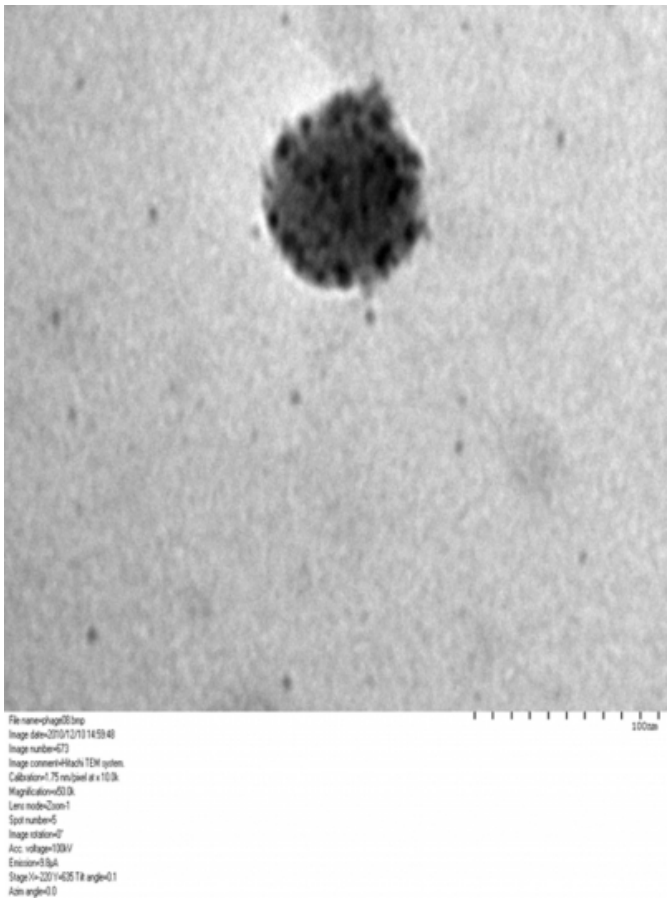
The host range of brucellaphage is against vaccine strains, viz. S19, S99 and Rev1 of B. melitensis, B. melitensis and B. suis. All the nine field isolates of B. abortus were sensitive to phage. The isolated brucellaphage failed to lyse any culture of heterologous species tested. Pasteurella multocida, E. coli, Staphylococcus, Streptococcus, Salmonella Dublin, Micrococcus and Pseudomonas spp. A broader host range of brucellaphage has been reported against all the three biotypes of B. melitensis as well as other species of Brucella by Douglas and Elberg, 1976.

## ELECTRON MICROSCOPIC FINDINGS

Electron microscopic studies of the brucellaphage revealed it to be an elementary body measuring approximately 65 nm at 50,000 X magnification with rounded head and a very short tail (Fig.3). The size and shape resembles another Brucella phage Tbilisi phage and the other phages isolated elsewhere. Electron microscopic findings are in agreement with those of Mc Duff et al 1961 who indicated the electron micrographs of the isolated brucellaphage to be 65 mμ in diameter, polygonal in shape with a short tail. Similarly, Zhu et al (2009) indicated that the Tbilisi phage consist of a elementary body with capsid of 57 ± 2 nm in diameter, a collar of 12 ± 2 nm and a 32 ± 3 nm contractile tail.

**Figure 3**

Fig 3 Electron microscopy of brucellaphage



## EFFECT OF VARIED TEMPERATURES ON THE BRUCELLAPHAGE

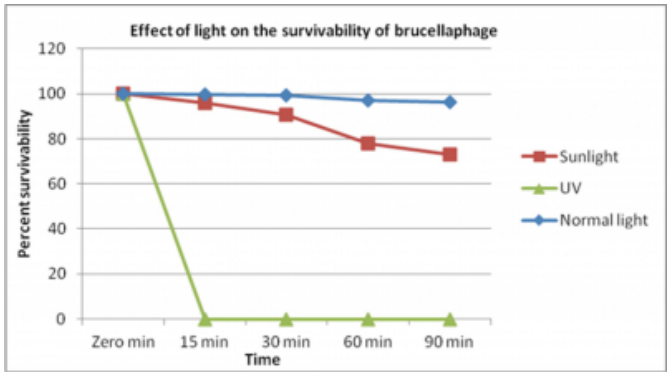
The phage was able to survive at temperatures of -20°C, 4°C, 37°C and 50°C when exposed for duration of 20 min. But no growth was observed when exposed to 70°C and 100°C for a similar time period of 20 min. Temperature of 70°C and beyond was lethal for the brucellaphage suggesting the wider range of adaptability of the phage in psychrophilic and mesophillic environments. On the other hand, higher temperature of 70°C and beyond is lethal which shows the intolerance to higher temperatures.

## EFFECT OF LIGHT ON THE BRUCELLAPHAGE

Survivability of brucellaphage gradually decreased from 99.8% (on exposure for 15 min) to 96.17% over a 90 min period in normal room temperature fluorescent light as shown in Fig. 4). Similarly, sunlight also gradually decreased the survivability of brucellaphage from 95.8% (on exposure for 15 min) to 73% over a 90 min period (Fig.4). However, UV light killed the brucellaphage within the first 15 min. Unlike normal fluorescent light, the effect of sunlight on the survivability of phage indicates deleterious effects on the phage and probably it is thus keeping a check on the virus in the environment. The UV light completely destroys the phage within 15 min. This property could be useful in disinfection of areas with UV.

### Figure 4

Fig 4 Effect of light on the survivability of brucellaphage

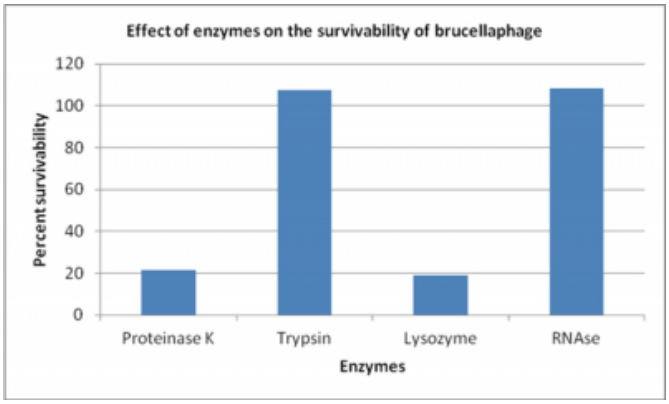



## EFFECT OF ENZYMES ON THE BRUCELLAPHAGE

Survivability of brucellaphage was 21.6% and 19.03% on exposure to proteinase K (20 mg/ml) and lysozyme (20 mg/ml) for 15 min., respectively. Survivability of brucellaphage was 107.33 % and 108.48% on exposure to trypsin (250µg/ml) and RNAse (10 mg/ml), respectively (Fig.5).

### Figure 5

Fig 5 Effect of enzymes on the survivability of brucellaphage




## EFFECT OF SDS (10%), NSS, EDTA (0.01M) ON THE BRUCELLAPHAGE

SDS (10%) completely destroyed the brucellaphage in 15 min. There was no change in the survivability of brucellaphage uptil 3 h incubation in NSS. Even 0.01 M EDTA treatments for 3 h did not affect the survivability of brucellaphage.

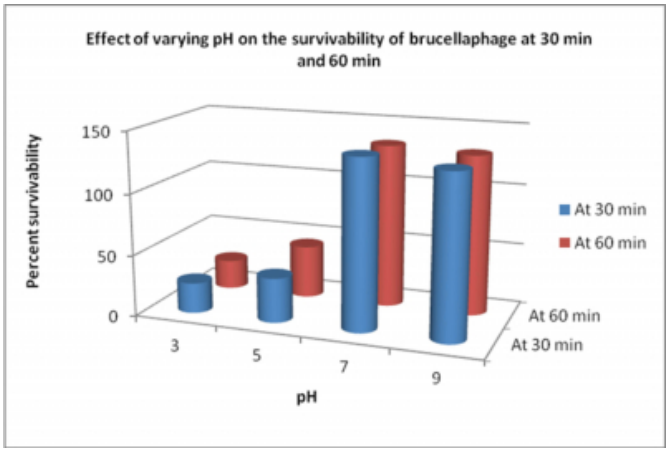
## EFFECT OF VARIED PH ON THE SURVIVABILITY OF BRUCELLAPHAGE

The survivability of brucellaphage varied from 24.77% (30 min exposure times) to 24.08% (60 min exposure times) at pH 3 (Fig.6). Similarly it was 36.24% and 43.12%, respectively at pH 5. However, at pH7 and pH9, the survivability was 137.61% and 131.88%, respectively (on exposure for 30 min) and 133.49% and 130.28%, respectively (on exposure for 60 min). The isolated phage was tolerant to pH 7 and 9 while there was a reduction in phage activity at pH 3 and 5, suggesting the lethal activity of acidic pH towards the phage.

Similar findings regarding the physicochemical characterization of isolated brucellaphage have also been reported by Mc Duff et al 1961, Calderone and Pickett (1965) and Corbel et al. 1988. According to the literature reviewed, this is the first report of isolation of a brucellaphage from Punjab.

**Figure 6**

Fig 6 Effcet of varying pH on the survivability of brucellaphage at 30 min and 60 min.

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