INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–1639/2021/25–5–1066–1074
DOI: 10.17957/IJAB/15.1765
http://www.fspublishers.org

Full Length Article

Isolation, Propagation and Biocontrol Activity of Indigenous Bacteriophages against *Brucella abortus*

Arfat Yousaf Shaheen[1], Ali Ahmad Sheikh[1*], Masood Rabbani[1], Wasim Shehzad[2], Zaigham Abbas[3] and Munazzah Maqbool[1]

[1]*Institute of Microbiology, University of Veterinary and Animal Sciences, Sheikh Abdul Qadir Jelani Road Lahore Pakistan*
[2]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Sheikh Abdul Qadir Jelani Road Lahore Pakistan*
[3]*Department of Microbiology & Molecular Genetics, Canal Bank Rd, Punjab University New Campus, Lahore Punjab*
[*]For correspondence: ali.ahmad@uvas.edu.pk
Received 17 November 2020; Accepted 18 January 2021; Published 16 April 2021

Abstract

Brucellosis, being a zoonotic disease, is difficult to control despite the availability of vaccines. Bovine brucellosis could be controlled effectively using brucellaphages. This study was conducted to isolate bacteriophages against *Brucella abortus* from cattle farms sewage/slurry samples (n=50). Isolation and propagation of brucellaphages was made through spot and plaque assay. Two samples (n=2) were found positive on the plates as circular, clear, and pinpoint plaques (0.3 to 0.5 mm) with 4.6×10^6 PFU/mL of brucellaphage titre against *Brucella abortus* RB51. One- step growth experiments revealed latent period of 120 min and burst size of 93 and 114 PFU for two isolated brucellaphages (ϕP1 and ϕP2) respectively. These phages were unable to lyse *Staphylococcus aureus*, *Streptococcus* spp., *Salmonella* spp., *Escherichia coli*, *Bacillus subtilis* and *Pasteurella multocida*. Isolated brucellaphages were stable up to 60°C and between 7 to 9 pH. No loss in phage titres were observed at 4°C but phage titres were reduced by one log at (-20°C) overnight. There was no effect of SM buffer, normal saline and EDTA on stability of brucellaphages and addition of divalent salts in medium showed significant increase in PFU. However, treatment with SDS and chloroform destroyed the phages in one hour exposure. The phages were found carrying double stranded DNA (~40 kb size) and two prominent protein bands of 45 kDa and 70 kDa. Biocontrol activity of brucellaphages showed average *Brucella abortus* count reduction to 4.5×10^3 from 5.0×10^8 CFU/ g of soil sample within 48 h when treated with maximum phage titre of 5.0×10^{11} PFU/ mL. Hence, this study revealed that brucellaphages are present in our environment and can potentially be consider for their cost-effective practical applications in inactivation of *Brucella abortus* after comprehensive experimental evaluations.

Keywords: Brucellosis; Brucellaphages; *Brucella abortus*; Overlay technique; Characterization; Biocontrol activity

Introduction

Bovine brucellosis is a significant bacterial disease with zoonotic potential and economic importance worldwide and is mainly caused by *Brucella abortus*. *B. abortus* is a small, non-motile, non-sporing, gram negative, facultative intracellular coccobacilli of the genus *Brucella*. *Brucella* is a member of the *Brucellaceae* family, in the order Rhizobiales and class Alphaproteo bacteria. Main routes of transmission of *Brucella* organism is through mucous membranes, ingestion and also from broken skin. Among various infectious diseases, brucellosis is one of the top-ranked bacterial diseases prevalent in developing countries. It is a highly contagious, chronic infectious disease in animals and among zoonotic diseases, it ranked at second position (OIE 2019). The most common consequences of bovine brucellosis are last trimester abortions, retained placenta, metritis, infertility, orchitis and epididymitis in animals, hence producing economic losses to livestock sector in terms of abortion, low fertility rate, decrease milk yield and loss due to replacement of the animal (Dean *et al*. 2012; Manish *et al*. 2013).

Being zoonotic, it can be transferred to humans through direct contact with infected animal material or indirectly by ingestion of contaminated dairy products. In countries like Pakistan, importance of the disease is more as majority of rural population is involved in livestock farming (Shafee *et al*. 2011). Seroprevalence of 18.6 and 8.7% was studied in herds and animals respectively in various districts of Pakistan (Ali *et al*. 2017; Arif *et al*. 2019). Efforts are made to control the problem in endemic areas by using two commercially available live vaccines (RB51 and S19), but these vaccines are not producing desirable results and being live vaccines, these cause abortions in pregnant animals and

To cite this paper: Shaheen AY, AA Sheikh, M Rabbani, W Shehzad, Z Abbas, M Maqbool (2021). Isolation, propagation and biocontrol activity of indigenous bacteriophages against *Brucella abortus*. *Intl J Agric Biol* 25:1066–1074

also have unwanted effects in humans exposed to the vaccines. The effective control of brucellosis is by eradication program. However, this procedure cannot be employed in developing countries like Pakistan due to high cost of animals (Cutler 2005).

Bacteriophages have been found to be potential candidates for prevention and treatment of many bacterial diseases. The phages are very specific to their host and only target specific bacteria and this specificity make them unique by not targeting human and other animal cells (Chachra *et al*. 2012; Filippov *et al*. 2013). Previous literature had studies regarding the bacteriophage-based diagnostics, phage typing, and epidemiological investigation of brucellaphages (Gupta and Saxena 2017a; Sergueev *et al*. 2017). Most of the studies were about the genetic diversity among previously characterized reference brucellaphages (Tb, Bk, R/C, Wb, Fi, Iz, Pr) but remains limited about the successful use of brucellaphages for specific decontamination and antibacterial therapy (Hammerl *et al*. 2017). Few studies have been concerned with the use of specific bacteriophage for prophylaxis and therapy against brucellosis (Chachra *et al*. 2012; Pandey *et al*. 2013; Prajapati *et al*. 2014; Jain *et al*. 2015; Gupta and Saxena 2017b; Saxena and Raj 2018). However, there is not any study conducted so far regarding the use of specific bacteriophage for the biocontrol of brucellosis with an idea of reducing *Brucella* contamination in livestock and dairy farms. The past studies encouraged us to isolate bacteriophages active against *B. abortus* in our country. So, objectives of our first study in Punjab, Pakistan were to isolate, propagate and evaluate biocontrol activity of bacteriophages against *B. abortus*, which may be fruitfully, applied in future studies of field applications of brucellaphages for the control of bovine brucellosis.

Materials and Methods

Location

This study was conducted at the University Diagnostic Laboratory (UDL), University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan from the year 2017–2019.

Samples

Samples of sewage water/slurry (n=50) were collected from *Brucella* suspected livestock farms, Punjab under strict safety measures recommended in OIE (2018). Out of 50 collected samples, 42 samples were of slurry (semi-liquid mixture of manure) and 8 were of sewage drainages of Lahore.

Host strain identification

Live vaccine of bovine *Brucella abortus* (RB51) was purchased from local veterinary drug store and was used as host strain, under strict biosafety precautions as per recommended in OIE (2019). Growth of *B. abortus* on solid media was obtained by inoculating 0.2 mL of vaccine (RB51) on Tryptose Soy Agar (TSA) (Merck Millipore, Germany) and incubated aerobically at 37°C for 3 to 5 days (Saxena and Raj 2018). To attain the sufficient bacterial turbidity ($O.D_{600}$=0.8), different conditions were tested included tube cultures and cultures in flasks by giving inoculation in test tube and conical Erlenmeyer flask. Liquid culture of *B. abortus* was obtained by inoculating the loopful culture (single colony) of RB51 strain from TSA plate in Tryptose Soy Broth (TSB) (Merck Millipore, Germany) in conical Erlenmeyer flask, filled as 1/5 of their nominal volume and incubated at 150 rpm, 37°C overnight (Sergueev *et al*. 2017). RB51 strain was identified based on its characteristic of resistance to rifampicin antibiotic by growing on rifampicin added TSA medium (250 μg/mL) and incubated at 37^{o}C for 72 h (OIE 2019). For further confirmation, PCR was performed for the *IS711* repetitive genetic region of bacterial genome. Bacterial DNA isolated using QIAamp DNA Mini kit (QIAGEN) was amplified as per method of (O'Leary *et al*. 2006). Thermocycler (BIO-RAD T100^{TM}) was programmed according to conditions as mentioned in Table 1.

Isolation and propagation of bacteriophages

Briefly, 50 mL 2X TSB, 40 mL slurry supernatant and 10 mL of broth culture of RB51 (log phase=24 h) having ($O.D_{600}$=0.8) were added and incubated at 37°C, 120 rpm for 10 days. 10 mL sample was drawn out on alternate days *i.e*., (2, 4, 6 and so on up to 10 days), and checked for presence of bacteriophages through spot assay (Chachra *et al*. 2012). Propagation of bacteriophages was carried out by giving successive enrichment to filtrates found positive in spotting, for three to four times with RB51 strain (Texas 2011). Subsequently, plaque assay was performed, using double agar overlay technique to observe plaques. Optimum adsorption time of phage with RB51 was determined by incubating the mixture of filtrate and RB51 at 37^{o}C at varied adsorption time (15, 30, 60, 90 and 120 min) before adding into soft agar containing 0.05 *M* $CaCl_2$ and $MgCl_2$ to the final concentrations. Semisolid TSB with 0.65% wach of agarose and bacteriological agar (maintained at 45°C in water bath) were used as soft agar for overlaying and both formulations were compared for good recovery of plaques on them (Yang *et al*. 2010).

Titration and purification of brucellaphages

Phage titre was measured through plaque assay by serially diluting the sterile phage filtrates to six different dilutions (10^{-1}–10^{-6}) in TSB as described by (Gupta and Saxena 2017a). Purification of phages was carried out by three-fold successive single plaque separation until homologous plaques were obtained (Hamza *et al*. 2016). Results for all

Table 1: Thermo cycler conditions for PCR

Stages	PCR conditions		Cycles
	Temp.	Time	
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	1.15 min	
Annealing	55.5°C	2 min	30
Extension	72°C	2 min	
Final extension	72°C	10 min	1

the experiments *i.e*., morphology of the plaques, host range, temperature and pH stability, molecular characterization and biocontrol activity were compared with the positive control brucellaphage strain, Tbilisi (Tb) phage obtained from Felix d' Herelle Reference Center for Bacterial Viruses (Laval University, QC, Canada) (Sergueev *et al*. 2017).

One-step phage growth curve and burst size

One-step growth experiments were conducted according to Wong *et al*. (2014) with modifications on time points and adsorption. A mid-log phase bacterial culture was infected with a phage suspension to a MOI ratio of 0.1. The mixture was incubated for 30 min at 37°C, with shaking at 120 rpm and was subsequently centrifuged at 7000 × g for 5 min. The supernatant was used for the determination of unabsorbed phage titre by the agar overlay assay. To determine the one-step growth kinetics of the phages, infected phages were obtained by centrifugation at 7000 ×g for 5 min at 4°C. The infected phages were resuspended in an equal volume of pre-warmed TSB medium and then incubated at 37°C with agitation. After every 30 min, for up to 4 h, the sample was withdrawn and assessed for phage titre using double agar overlay assay. The phage titres were then plotted against time intervals (Wong *et al*. 2014).

Heterogeneous bacterial species specificity of brucellaphages

Host range of isolated brucellaphages (n=2 positive) (ϕP1 and ϕP2) was checked against heterogeneous bacterial species of veterinary importance *viz*., *Pasteurella multocida* (ATCC® 43137™), *Staphylococcus aureus* (ATCC® 23235™), *Streptococcus sp.* (ATCC® 9884™), *E. coli* (ATCC® 25922™), *Salmonella spp.* (ATCC® 35664™) and *Bacillus subtilis* (ATCC® 23857™) (Chachra *et al*. 2012).

Stability of brucellaphages

Thermal stability of phages (ϕP1 and ϕP2) was checked by incubating phage suspensions in TSB (pH=7.0) at various temperatures (25, 37, 45, 60, 70, 80, 90°C) for one hour. Stability of phages at low temperatures *i.e*., - 20°C and 4°C was observed overnight. pH stability of phages (ϕP1 and ϕP2) was determined by incubating phage suspensions in TSB, adjusted in steps of 1 pH unit from pH 2 to 9, for one hour at 37°C. Effect of treatment with organic solvents was studied by incubating phage suspension with an equal volume of Sodium Dodecyl Sulphate (SDS) (10%), chloroform and EDTA (0.01 *M*) for one hour at 37°C. Effect of osmotic shock and inorganic salts was evaluated by incubating the phage suspensions in Saline Magnesium (SM) buffer (100 m*M* NaCl, 25 m*M* Tris-HCl pH 7.5, 8 m*M* $MgSO_4$) and 0.5 molar concentrations of Sodium chloride (NaCl), Calcium chloride ($CaCl_2$), and Magnesium chloride ($MgCl_2$) respectively overnight at 37°C. Phage-free and bacterial-free suspensions were used as controls incubated under the same conditions as the phage-bacterial suspensions (Chachra *et al*. 2012; Hamza *et al*. 2016). Subsequently, phage titres were checked by double agar overlay method.

Brucellaphages genome and proteins characterization

Phage genome was extracted by PCI (phenol-chloroform-isoamyl alcohol) method. Extracted genome was subjected to digestion with DNase I, RNase A and S1 nuclease (Thermo Scientific, U.S.A.) (Zhu *et al*. 2009). Restriction endonuclease analysis was carried out by incubating about 1 μg of each DNA sample with approximately 1 μL of FastDigest™ restriction enzymes *Hind*III and *Eco*RI (Thermo Fisher Scientific, U.S.A.) for 2 h at 37°C (Wang *et al*. 2018). The purified phage preparation (1×10^{10} PFU/mL) and the host strain were analyzed for proteins characterization through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Zhu *et al*. 2009).

Evaluation of biocontrol activity of brucellaphages

Biocontrol activities of brucellaphages were determined against live cultures of *B. abortus* RB51 and *B. abortus* S99 strains mixed in farm soil in sham (lab-based field conditions). For sham experiment, soil sample (100 g) from dairy farm was collected and sterilized through autoclave before inoculating it with 20 mL volume of broth culture of bacteria in log phase containing 5×10^8 CFU/mL of *B. abortus* strains and treated with ϕP1 and ϕP2 phages. Immediately after bacterial inoculation, phage suspensions with specified titres *i.e*., 5×10^{11}, 5×10^8, and 5×10^5 PFU/mL for each of phage were tested for bacterial count reductions by adding in bacteria inoculated soil sample. Pre and post treatment counts of *B. abortus* in farm soil were estimated through viable plate count. Bacterial count reduction was observed at 12, 24, 36, 48 and 72 h time points at 37°C. Tb phage was tested as control phage with same conditions applied for ϕP1 and ϕP2. Phage-free and bacterial-free suspensions were used as controls incubated under the same conditions as the bacteria-phage treated soil samples.

Statistical analysis

Results for two assays (Spot and Plaque) were compared

through McNemar Test, and one-way analysis of variance (ANOVA), with 95% confidence interval was used for comparing temperature and pH stability of phages as well as for biocontrol activity by using statistical package for social sciences (S.P.S.S.) version 23.0.

Results

Pure culture of *B. abortus* with isolated colonies (round, small, translucent and a pale honey colour colonies of 0.5 mm diameter) was observed on TSA appeared after 72 h of incubation. It was observed that turbidity in test tube was 0.2 at $O.D_{600}$ in TSB at 37°C up to 48 h but it was enhanced to $O.D_{600}$ = 0.8 with TSB in 24 h at 37°C and shaking at 150 rpm in conical Erlenmeyer flask. Growth of RB51 strain was found on TSA plates containing rifampicin antibiotic. Upon further confirmation on molecular basis through PCR, product (amplicon) size of 498 bp for IS711 gene for *B. abortus* was observed (Fig. 1).

Seven samples were found positive in screening (spot method) as clear zones of lysis apparent on the plates indicated the presence of viable bacteriophages against *B. abortus* (Fig. 2) and two of the seven screened samples gave positive plaques in plaque assay on the 6th day of incubation. Out of these 7 positive samples, 1 sample was found positive from sewage samples (n=8) tested and remaining 6 positive samples were of slurry samples (n=42) of livestock farms. In our first attempt of plaque assay, pinpoint and hazy plaques of bacteriophages were observed with soft agar (TSB containing 0.65% bacteriological agar) (Fig. 3a). However, in second attempt of using soft agar preparation with 0.65% agarose, we obtained the clear pinpoint plaques of diameter 0.5 mm as compared to soft agar preparation with 0.65% bacteriological agar (Fig. 3b).

Plaque morphology of isolated brucellaphages (n=2) was similar to that for Tb phage. Tb phage produced pinpoint, round and clear plaques of 0.5 mm diameter but reached maximum of 2.0 mm after 48 h of incubation with *B. abortus.* Optimum adsorption time for the brucellaphages was determined to be 120 min in this study as maximum plaques (4.5×10^{-6} PFU/mL) were recovered in 120 min. Results for two assays (Spot and Plaque) were compared, and statistically non-significant 0.062 ($P > 0.05$) value was obtained indicating that there is no significant difference between plaque assay and spot assay. The latent time of ϕP1, ϕP2, and Tb was estimated to be 120, 120 and 90 min respectively, while the burst size of these phages was 93, 114 and 122 PFU per infected cell, respectively (Fig. 4).

Host range of brucellaphages revealed that they are unable to lyse heterologous bacterial species. Testing of the thermal stability of ϕP1 and ϕP2 phages revealed their viability up to 60°C but when exposed to 70°C, their viability starts decreasing (Fig. 5). In comparison, Tb phage had stability up to the 80°C but beyond 80°C it was inactivated in one hour exposure. It was observed that refrigeration temperature (4°C) did not affect the plaque

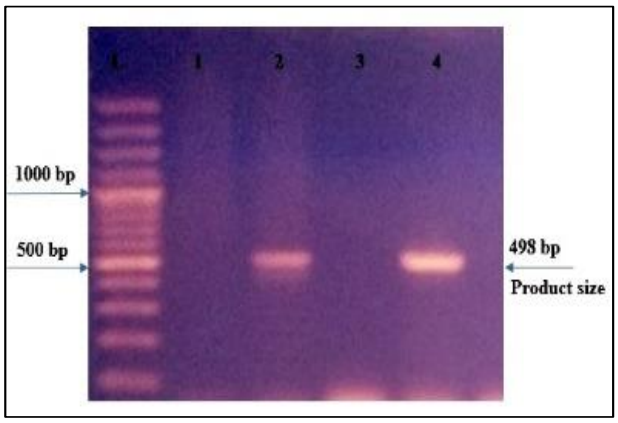

Fig. 1: Molecular identification of *B. abortus* through PCR, L: 100 bp DNA Ladder, 1: negative control, 2: RB51 vaccine DNA, 3: negative sample 4: RB51 culture DNA

Fig. 2: Zones of Lysis showing positive spot test for suspected phages against *B. abortus* RB51

formation of phages as well as no loss in titres of phages was observed at 4°C. While, one log reduction in titre was observed at freezing temperature (-20°C) overnight. When a set of temperatures were analyzed statistically using univariate analysis of variance (ANOVA), non-significant difference (P= 0.14, 0.67, 0.06) was found in comparison of 25, 37, 45 and 60°C, respectively while significant (P=0.00) difference was observed when compared higher temperatures of 70 and 80°C. pH stability of phages showed that present study brucellaphages have stability between pH 7 to 9, but acidic pH of 2 to 4 decreased the phage viability in one hour exposure (Fig. 6). Significant difference (P=0.00) was present in comparison of acidic and basic pH values using ANOVA. There was no reduction in phage titres was observed in SM buffer and viability was

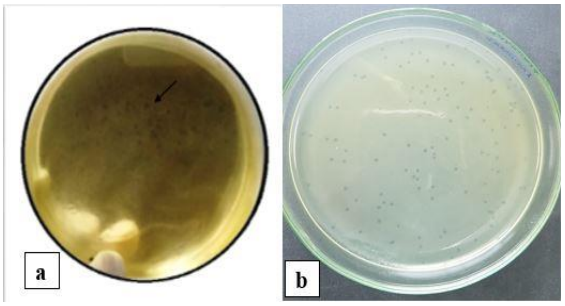

Fig. 3: Positive plaques of bacteriophages against *B. abortus* RB51. (**a**); pinpoint, hazy plaques of brucellaphages from first enrichment of slurry sample with *B. abortus* RB51 after incubation of 48 h at 37°C, (**b**); pinpoint, clear plaques of ϕP1 after the addition of $CaCl_2$ and $MgCl_2$ in soft agar, successive enrichment of 3 to 4 times and use of 0.65% agarose for soft agar and incubation of 48 h at 37°C

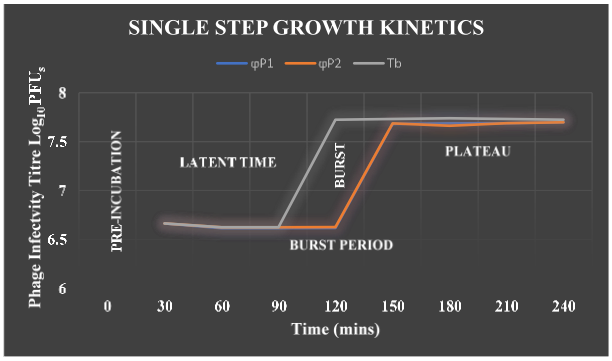

Fig. 4: One-step growth curves of brucellaphages. (**a**); ϕP1 (**b**); ϕP2 and (**c**); Tb

maintained overnight. No effect on viability of brucellaphages was observed after treatment with saline solution and EDTA. However, treatment with SDS and chloroform destroyed the brucellaphages in one hour exposure. Influence of inorganic salts on phages, showed a significant increase in PFU from 3.9×10^6 to 4.5×10^6 after the incorporation of $CaCl_2$ and $MgCl_2$ in the medium containing phage suspension. NaCl neither enhanced the plaque size nor lessened the PFU, showing neither a positive nor a negative effect on phage titre. Significant difference (P=0.00) was observed in phage viability when treated with saline solution and EDTA in comparison with SDS and chloroform.

Nucleic acid extracts of theses phages were found resistant to RNase A and S1 nuclease but have sensitivity to DNase I enzyme. The genome sizes of phages were approximately 40 kb for two isolated brucellaphages and Tb phage (Fig. S1). *Hind*III digestion of the three phages depicted the two extra bands in Tb phage while the corresponding bands of ~1000 bp and ~1800 bp are absent in present study phages. *Eco*RI digestion of phages showed one extra band in Tb phage however other two phages have similar band patterns (Fig. S2). Characterization of proteins of isolated brucellaphages and Tb phage through SDS-PAGE showed coomassie-stained bands of different sizes. The most prominent bands were of 45 kDa and 70 kDa in tested brucellaphages (Fig. S3).

Biocontrol activities of brucellaphages against live cultures of *B. abortus* RB51 and *B. abortus* S99 strains mixed in farm slurry in sham conditions showed the reduction in bacterial count at 24, 36, 48 and 72 h time points (Table 2). This count was reduced to 10^7, 10^5, and 10^3 CFU/g after 24, 36 and 48 h treatment respectively. Negligible bacterial count reduction was observed before 12 h and count reduction became static after 72 h of treatment. Significant biocontrol activity (P-value < 0.05) was observed among three tested phages with specified titres in all treatments using ANOVA in three replicate experiments.

Discussion

The presence of bacteriophages against *B. abortus* was studied in several countries which established a close relationship between sensitivity of *B. abortus* cultures to lysis by phages. They used field isolates as well as vaccine strains of *B. abortus* in their studies (Zhu *et al*. 2009; Pandey *et al*. 2013; Farlow *et al*. 2014; Hammerl *et al*. 2014; Tevdoradze *et al*. 2015; Gupta and Saxena 2017a; Hammerl *et al*. 2017). Therefore, it is not astonishing to find lytic bacteriophages against *B. abortus* worldwide. In this study, we first established the optimized growth conditions for the host bacterial culture. For *B. abortus*, results of present study, declared that the use of conical Erlenmeyer flask instead of glass tube culture and shaking at 150 rpm were critical for sufficient turbidity of *B. abortus*. The results showed that increased turbidity ($O.D_{600}$= 0.8) in flask could be due to shaking which provides aeration and splitting of bacterial clumps in the broth. Increased bacterial turbidity within 24 h could be advantageous to avoid phage resistance to host strain and for efficient phage propagation. Our observation of increased bacterial turbidity with increased aeration is correlated with Wundt (1957) observation, that growth of *Brucella* was greatly retarded by inefficient gas exchange when cultures were contained in 'tubes' though several liquid media were checked for the growth promoting capacity for *Brucella*. Gibby and Gibby (1964), also suggested that if active growth phase of *Brucella* is desired then its growth should be harvested before 24 h and counts of *B. abortus* growth declined slightly from 48 to 96 h. Similarly, previous experiments of Gee and Gerhardt (1946), showed that the use of aerated liquid media, generation time of *Brucella* decreased as aeration was increased until aeration reached 2 volumes per min (volume of air to the volume of media).

In the present study, Tb phage produced pinpoint, round and clear plaques of 0.5 mm diameter but reached maximum of 2.0 mm after 48 h of incubation. Plaque morphology of present study brucellaphages (ϕP1 and ϕP2) was same as of Tb phage *i.e*., pinpoint, round and clear

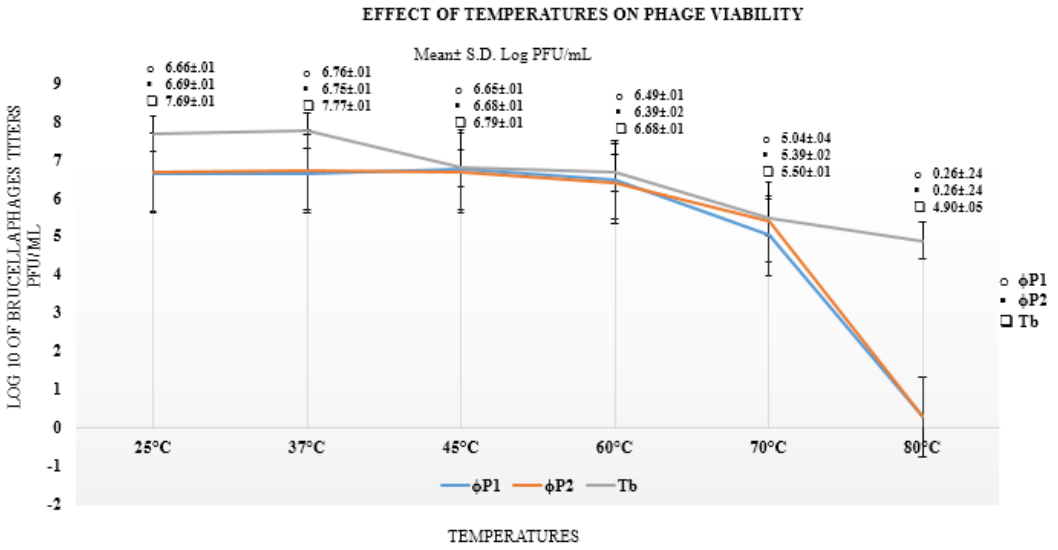

Fig. 5: Graphical representation of thermal stability of brucellaphages. Phage suspensions in TSB (pH=7.0) were incubated for one hour at adjusted temperatures. Post treatment viability was assessed through double agar overlay technique. Log_{10} values of phage titres (y-axis) were plotted against various temperatures (x-axis) for phage isolate 1 (ϕP1), phage isolate 2 (ϕP2) and positive control Tbilisi phage (Tb). Data represent means ± SD (standard deviation) of three independent experiments with similar results

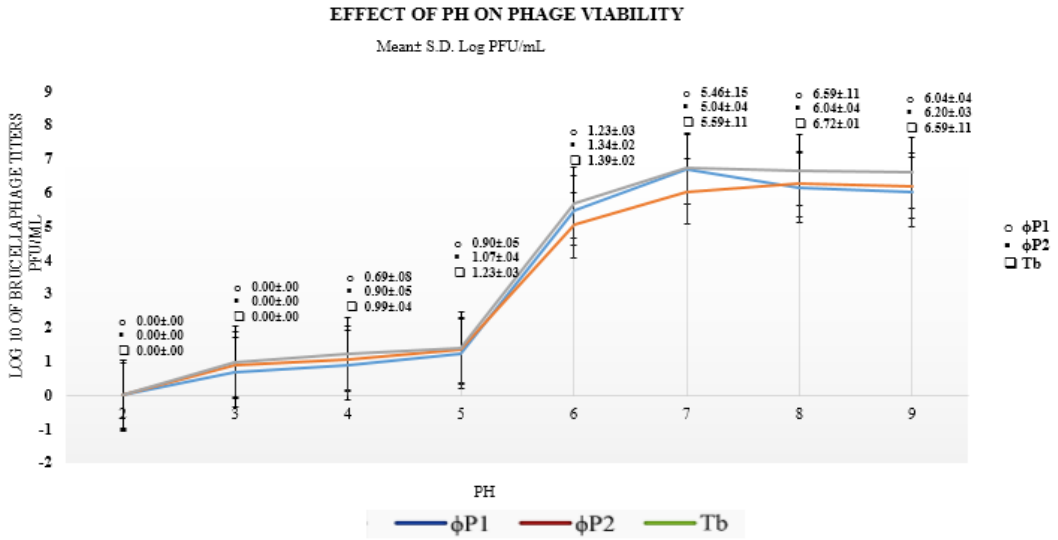

Fig. 6: Graphical representation of stability of brucellaphages at acidic and basic pH values. Phage suspensions in TSB adjusted in pH range 2 to 9 were incubated for one hour at 37°C. Post treatment viability was assessed through double agar overlay technique. Log_{10} values of phage titres (y-axis) were plotted against different pH values (x-axis) for phage isolate 1 (ϕP1), phage isolate 2 (ϕP2) and positive control Tbilisi phage (Tb). Data represent means ± SD (standard deviation) of three independent experiments with similar results with 95% confidence interval

plaques of 0.5 mm diameter. Both phages (ϕP1 and ϕP2) have identical plaque morphology (round, clear and pinpoint plaques having 0.5 mm diameter) after 48 h of incubation at 37°C. However, they were different in isolation source/sample nature *i.e.*, one sample was found positive from slurry of livestock farm and other from sewage sample. Chachra *et al*. (2012); Pandey *et al*. (2013); Gupta and Saxena (2017b) and Saxena and Raj (2018) also found circular and clear plaques of 0.1–3.0 mm diameter of brucellaphages in their studies. In the present study brucellaphages were isolated from slurry samples of dairy farms on the 6th day of incubation. It was attempted to isolate phages from the day 1 to day 5 but there was no indication of phage in sample. This finding is correlated with study of Chachra *et al*. (2012) and Pandey *et al*. (2013), in which they isolated brucellaphage from sewage sample of dairy farm on

Table 2: Biocontrol activity of brucellaphages in sham experiment

Bacterial target inoculum (CFU/mL)	Phage	Phage titre tested (PFU/mL)	Bacterial count reduction in (CFU/g) soil ± standard deviation with time intervals				
			12 h	24 h	36 h	48 h	72 h
B. abortus RB51- 5.0×10^8	ϕP1	5.0×10^{11}	$4.6 \times 10^8 \pm .009$	$5.1 \times 10^7 \pm .008$	$6.8 \times 10^5 \pm .006$	$4.5 \times 10^3 \pm .009$	$4.3 \times 10^3 \pm .010$
		5.0×10^8	$4.7 \times 10^8 \pm .009$	$5.4 \times 10^7 \pm .008$	$7.3 \times 10^5 \pm .005$	$4.5 \times 10^3 \pm .009$	$4.5 \times 10^3 \pm .009$
		5.0×10^5	$4.7 \times 10^8 \pm .009$	$4.4 \times 10^8 \pm .009$	$3.8 \times 10^7 \pm .011$	$7.4 \times 10^6 \pm .005$	$7.1 \times 10^6 \pm .006$
B. abortus RB51- 5.0×10^8	ϕP2	5.0×10^{11}	$4.7 \times 10^8 \pm .009$	$6.1 \times 10^7 \pm .039$	$7.2 \times 10^5 \pm .020$	$4.8 \times 10^3 \pm .009$	$4.7 \times 10^3 \pm .009$
		5.0×10^8	$4.9 \times 10^8 \pm .008$	$5.5 \times 10^7 \pm .007$	$7.5 \times 10^5 \pm .005$	$5.0 \times 10^3 \pm .008$	$4.7 \times 10^3 \pm .009$
		5.0×10^5	$5.0 \times 10^8 \pm .008$	$4.5 \times 10^8 \pm .009$	$4.0 \times 10^7 \pm .010$	$7.9 \times 10^6 \pm .005$	$7.6 \times 10^6 \pm .005$
B. abortus S99- 5.0×10^8	Tb	5.0×10^{11}	$4.1 \times 10^8 \pm .010$	$4.9 \times 10^7 \pm .008$	$6.3 \times 10^5 \pm .006$	$4.0 \times 10^3 \pm .010$	$3.8 \times 10^3 \pm .011$
		5.0×10^8	$4.3 \times 10^8 \pm .010$	$4.8 \times 10^7 \pm .009$	$6.9 \times 10^5 \pm .006$	$4.2 \times 10^3 \pm .010$	$4.1 \times 10^3 \pm .010$
		5.0×10^5	$4.3 \times 10^8 \pm .010$	$4.1 \times 10^8 \pm .010$	$3.4 \times 10^7 \pm .012$	$7.1 \times 10^6 \pm .006$	$6.9 \times 10^6 \pm .006$

day sixth of incubation with actively growing stage of *B. abortus*. This could be because of the long generation time of the bacteria *i.e*., 4 h in liquid culture approximately as also reported by (McDuff *et al*. 1962).

Isolation and propagation of bacteriophages for *B. abortus* using spot assay method and double agar overlay technique for plaques in this study demonstrated that initial spot and plaque assays gave the evident positive results in enriched samples. It was revealed that bacteriophages were present and were able to infect *B. abortus* as lysis was apparent on the plates in our environment. Successive enrichment of three to four times, varying adsorption time, and addition of $CaCl_2$ and $MgCl_2$, we obtained the clear pinpoint plaques of brucellaphages. These results are in agreement with Chhibber *et al*. (2014) and Rasool *et al*. (2016) indicating the crucial importance of divalent ions, successive enrichment and adsorption time in adsorption of phage to the host cell surface. Adsorption time determined for present study phages corresponds to Tevdoradze *et al*. (2015) and Antadze *et al*. (2017) findings. Results for one-step growth kinetics of present study phages are in line with Jones *et al*. (1968) and McDuff *et al*. (1962).

Our observations for host range of present study brucellaphages indicated that they could not lyse heterogeneous bacterial species *i.e*., *S. aureus*, *Streptococcus sp*., *Salmonella sp*., *E. coli*, *Bacillus subtilis*, and *P. multocida*. Similar findings were demonstrated by Pandey *et al*. (2013) that their brucellaphage could not lyse any of the heterologous bacteria. Prajapati *et al*. (2014) also showed lytic activity of phage against *B. abortus* strain 99, S19 and 544 as well as *B. melitensis* Rev 1 and *B. suis* 1330, but did not show lysis against any of the heterogeneous bacterial species. Hence, these results determined that brucellaphage is specific for *Brucella* and not to other gram positive and gram-negative bacteria which is advantageous that other microflora in the body might not be disturbed.

In this study, stability of brucellaphages showed that their viability decreased when exposed to 70°C and beyond 70°C it completely became inactivated in one-hour exposure. They had stability at pH 7 to 9, but acidic pH of 2 to 4 decreased the phage viability in one-hour exposure. Stability of tested brucellaphages depicted that they retain their viability at 4°C but freezing temperature (- 20°C) were not revealed to be suitable for storage of phages as they lost their viability. Our results for temperature and pH stability of brucellaphages are correlated with Pandey *et al*. (2013) who found stability of brucellaphage at basic pH, i.e. pH 8 with survival rate of 75.31% after 48 h treatment. They also observed that at acidic pH of 2 to 4, the phage titre was gradually decreased to zero and at pH 6 phage titre was decreased only to 38.9% within 48 h. Gupta and Saxena (2017b) also studied inactivation of phage at pH 2 and 4 after 4 and 12 h treatment. Our findings are also supported by Chachra *et al*. (2012) in favor of pH and temperature stability that high temperature of 70°C and acidic pH is lethal for brucellaphage viability. Evaluation of thermal stability and optimum pH conditions of brucellaphages are helpful to standardize the phage therapy as well as bio decontamination. Extreme resistance to temperature is advantageous for brucellaphages to apply in field conditions to keep phages working in harsh conditions.

Genome characteristics of our brucellaphages showed their nucleic acid to be double-stranded DNA of 40 kb size, resistant to RNase A and S1 nuclease but have sensitivity to DNase I enzyme. Restriction profile of ϕP1 and ϕP2 revealed that they have different migration patterns *i.e*., ϕP2 moved slower than ϕP1. This might be due to higher molecular weight of phage 2. These characteristics are supported by Zhu *et al*. (2009); Farlow *et al*. (2014); Hammerl *et al*. (2014); Tevdoradze *et al*. (2015) and Hammerl *et al*. (2017). Results of restriction of two phages revealed that they are different from positive control brucellaphage Tb. The restriction endonuclease profiles were highly reproducible and consistent with phage Tb. Restriction analysis of study phages ascertained that our brucellaphage isolates are closely related but they are different from Tb phage. Structural protein profile of present study phages by SDS-PAGE revealed two prominent bands of 45 kDa and 70 kDa, which probably represents the major capsid proteins. Zhu *et al*. (2009) depicted nine bands, ranging from 40 to 85 kDa of the structural proteins of Tbilisi phage. Similarly, Pandey *et al*. (2013), observed 4 bands of 65.98, 60.46, 48.56 and 43.97 kDa proteins in brucellaphage. Isolated phages need to be further characterized particularly protein segments of the isolated phages need to be investigated for their antibacterial ability against *Brucella* so that they may be used in the

future for commercial lysate preparations. Biocontrol activity of our brucellaphages against live cultures of *B. abortus* RB51 and *B. abortus* S99 strains mixed in farm slurry in sham conditions showed significant reduction in bacterial counts when tested three of their titres. This count was reduced to 10^7, 10^5 and 10^3 CFU/g after 24, 36 and 48 h treatment, respectively. Negligible bacterial count reduction was observed before 12 h and this might be due to longer generation time of *Brucella* (4 h) and log phase of 24 h. Count reduction became static after 72 h of treatment. Plateau in reduction after 48 h might be due resistance or stability issues of phages which is an inherit limitation of our study. It needs to be investigated in future studies. Conferring literature reviewed, there is no such study conducted so far about the investigation of biocontrol activity of brucellaphages *in vitro*. Present study phages hold good antibacterial efficacy and can serve as biocontrol agents for the purpose of specific decontamination of *Brucella.* However, some issues remain to be controlled and studied as limitations of the present study such as the presence of toxic agents including endotoxin in the phage preparations, phage cocktails, stability and viability deficiencies, and the problem of bacterial resistance against phages.

Conclusion

In conclusion, this study provides base line for the investigation of indigenous bacteriophages for the field strains of *B. abortus* in laboratory in Pakistan. The isolated brucellaphages themselves have considerably more potential for further characterization. Therefore, more comprehensive studies are suggested in the future those might be molecular and genetic characterization including sequencing of phages, transmission electron microscopy, and *in vitro* and *in vivo* experimental evaluation of the isolated brucellaphages. Further studies could be designed to observe the bacterial resistance against the bacteriophages, needing a cocktail of bacteriophages to cover wide range of *Brucella* strains, presence of endotoxin in the phage preparations and stability and viability deficiencies. Subsequently, these bacteriophages can be used for the effective treatment of *Brucella* contaminated soil and environment of livestock and dairy farms, cost-effective diagnostics, and therapy of brucellosis using bacteriophages in different novel composition. Moreover, it will reduce the economic losses due to this deadly disease of dairy sector.

Acknowledgment

The authors gratefully acknowledge the financial support provided by Higher Education Commission (HEC) project # 6782/Punjab/NRPU/R&D/HEC/2016 under National Research Program for Universities, Pakistan and Denis Tremblay, (University Laval, Quebec City, QC, Canada) for the provision of positive control phage.

Author Contributions

AAS, ZA, MR and WS conceived and designed the study. AYS and AAS executed the experiments and analyzed the study results. MM helped in research work. All authors critically revised the manuscript for important intellectual contents and approved the final version.

Conflicts of Interes

The authors declare that they have no conflict of interest.

Data Availability

The data will be made avaialble on acceptable requests to the corresponding author.

Ethics Approval

Not applicable**.**

References

Ali S, S Akhter, H Neubauer, F Melzer, I Khan, EN Abatih, H El-Adawy, M Irfan, A Muhammad, MW Akbar, S Umar (2017). Seroprevalence and risk factors associated with bovine brucellosis in the Potohar Plateau, Pakistan. *BMC Res Notes* 10; Article 73

Antadze I, M Dadunashvili, T Burbutashvili, S Gunia, N Balarjishvili, E Tevdoradze, T Pataridze, RJ Obiso, S Hagius, P Elzer, M Kutateladze (2017). Diversity of phage-host specificity in *Brucella* Phage. *J Bacteriol Mycol* 4; Article id1049

Arif S, PC Thomson, M Hernandez-Jover, DM McGill, HM Warriach, K Hayat, J Heller (2019). Bovine brucellosis in Pakistan; an analysis of engagement with risk factors in smallholder farmer settings. *Vet Med Sci* 5:390–401

Chachra D, H Kaur, M Chandra, H Saxena (2012). Isolation, electron microscopy and physicochemical characterization of a brucellaphage against *Brucella abortus* vaccine strain S19. *Intl J Microbiol* 10; Article 2c47

Chhibber S, T Kaur, S Kaur (2014). Essential role of calcium in the infection process of broad-spectrum methicillin-resistant *Staphylococcus aureus* bacteriophage. *J Basic Microbiol* 54:775–480

Cutler SJ, AM Whatmore, NJ Commander (2005). Brucellosis–new aspects of an old disease. *J Appl Microbiol* 98:1270–1281

Dean AS, L Crump, H Greter, E Schelling, J Zinsstag (2012). Global burden of human brucellosis: A systematic review of disease frequency. *PLoS Negl Trop Dis* 6; Article e1865

Farlow J, AA Filippov, KV Sergueev, J Hang, A Kotorashvili, MP Nikolich (2014). Comparative whole genome analysis of six diagnostic brucellaphages. *Gene* 541:115–122

Filippov AA, KV Sergueev, MP Nikolich (2013). Bacteriophages against biothreat bacteria: Diagnostic, environmental and therapeutic applications. *J Bioterr Biodef* 3; Article S3-010

Gee LL, P Gerhardt (1946). *Brucella suis* in aerated broth culture: II. aeration studies. *J Bacteriol* 52:271–281

Gibby IW, AM Gibby (1964). Population dynamics of *Brucella* on solid media. *Appl Environ Microbiol* 12:442–446

Gupta V, HM Saxena (2017a). A new bacteriophage based luminescence assay for diagnosis of brucellosis. *Ind J Exp Biol* 55:296–302

Gupta V, HM Saxena (2017b). Isolation and characterization of BpL1, a broad acting lytic bacteriophage against *Brucella*. *Intl J Curr Microbiol Appl Sci* 6:2486–2496

Hammerl JA, C Gollner, C Jackel, HC Scholz, K Nockler, J Reetz, SA Dahouk, S Hertwig (2017). Genetic diversity of *Brucella* reference and non-reference phages and its impact on *Brucella*-typing. *Front Microbiol* 8; Article 408

Hammerl JA, SA Dahouk, K Nockler, C Gollner, B Appel, S Hertwig (2014). F1 and tbilisi are closely related brucellaphages exhibiting some distinct nucleotide variations which determine the host specificity. *Genome Announc* 2; e01250-13

Hamza A, S Perveen, Z Abbas, SU Rehman (2016). The lytic SA phage demonstrates bactericidal activity against mastitis causing *Staphylococcus aureus*. *Open Life Sci* 11; Article e00917

Jain L, M Rawat, A Prajapati, AK Tiwari, B Kumar, V Chaturvedi, H Saxena, S Ramakrishnan, J Kumar, P Kerketta (2015). Protective immune-response of aluminium hydroxide gel adjuvanted phage lysate of *Brucella abortus* S19 in mice against direct virulent challenge with *B. abortus* 544. *Biologicals* 43:369–376

Jones LM, G Merz, J Wilson (1968). Phage typing reactions on *Brucella* species. *Appl Environ Microbiol* 16:1179–1190

Manish K, C Puran, C Rajesh, R Teena, K Sunil (2013). Brucellosis: An updated review of the disease. *Ind J Anim Sci* 83:3–16

McDuff CR, LM Jones, J Wilson (1962). Characteristics of brucellaphage. *J Bacteriol* 83:324–329

O'Leary S, M Sheahan, T Sweeney (2006). *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. *Res Vet Sci* 81:170–176

OIE (2019). *Brucellosis (Brucella abortus, B. melitensis and B. suis) (infection with B. abortus, B. melitensis and B. suis).* Manual of diagnostic tests and vaccines for terrestrial animalshttp://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.01.04_BRUCELLOSIS.pdf

OIE (2018). *Biosafety and Biosecurity: Standard for managing biological risk in the veterinary diagnostic laboratories and animal facilities*. Manual of diagnostic tests and vaccines for terrestrial animals. https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.04_BIOSAFETY_BIOSECURITY.pdf

Pandey S, D Chachra, M Chandra, H Saxena (2013). Protein profiling and physico-chemical characterization of an isolated phage against *Brucella abortus* strain 19. *Afr J Microbiol Res* 7:1233–1238

Prajapati A, D Ramchandran, H Verma, M Abbas, M Rawat (2014). Therapeutic efficacy of *Brucella* phage against *Brucella abortus* in mice model. *Vet World* 7:34–37

Rasool MH, R Yousaf, AB Siddique, M Saqalein, M Khurshid (2016). Isolation, characterization, and antibacterial activity of bacteriophages against methicillin-resistant *Staphylococcus aureus* in Pakistan. *Jundishapur J Microbiol* 9; Article e36135

Saxena HM, S Raj (2018). A novel immunotherapy of brucellosis in cows monitored non invasively through a specific biomarker. *PLoS Negl Trop Dis* 12; Article e0006393

Sergueev KV, AA Filippov, MP Nikolich (2017). Highly sensitive bacteriophage-based detection of *Brucella abortus* in mixed culture and spiked blood. *Viruses* 9; Article 144

Shafee M, M Rabbani, AA Sheikh, A Razzaq (2011). Prevalence of *Bovine brucellosis* in organized dairy farms, using milk ELISA, in Quetta City, Balochistan, Pakistan. *Vet Med Intl* 2011; Article 358950

Tevdoradze E, J Farlow, A Kotorashvili, N Skhirtladze, I Antadze, S Gunia, N Balarjishvili, L Kvachadze, M Kutateladze (2015). Whole genome sequence comparison of ten diagnostic *Brucella*phages propagated on two *Brucella abortus* hosts. *Virol J* 12; Article 66

Texas (2011). Protocol: Phage enrichments. Center for Phage Technology Texas A&M University, College Station, TX 77843. https://cpt.tamu.edu/wordpress/wp-content/uploads/2011/12/Phage-enrichments-07-12-2011.pdf. (Accessed: April 4, 2016, last updated December 7, 2011)

Wang T, D Wang, Y Lyu, E Feng, L Zhu, C Liu, Y Wang, X Liu, H Wang (2018). Construction of a high-efficiency cloning system using the Golden Gate method and I-SceI endonuclease for targeted gene replacement in *Bacillus anthracis*. *J Biotechnol* 271:8–16

Wong CL, CC Sieo, WS Tan, N Abdullah, M Hair-Bejo, J Abu, YW Ho (2014). Evaluation of a lytic bacteriophage, Φ st1, for biocontrol of *Salmonella enterica* serovar Typhimurium in chickens. *Intl J Food Microbiol* 172:92–101

Wundt W (1957). Untersuchungen zur Entwicklung leistungsfahiger brucellennahrboden. *Zentralbl Bakteriol Orig* 165:393–402

Yang H, L Liang, S Lin, S Jia (2010). Isolation and characterization of a virulent bacteriophage AB1 of *Acinetobacter baumannii*. *BMC Microbiol* 10; Article 131

Zhu CZ, HY Xiong, J Han, BY Cui, DR Piao, YF Li, H Jiang, Q Ren, XY Ma, YM Chai, X Huang, HY Zhao, LY Li (2009). Molecular characterization of Tb, a new approach for an ancient brucellaphage. *Intl J Mol Sci* 10:2999–3011