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Research Article

Non - Invasive Monitoring and Assessment of Phage Therapy and Phage Targeting in Bovine Brucellosis Employing a *Brucella* - Specific Biomarker

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Abstract

Brucellosis caused by *Brucella* organisms is a major zoonosis globally. It causes heavy losses through abortions, delayed conception and infertility in animals. Antibiotic therapy is ineffective. Once infected, the animal remains carrier and sheds bacteria in milk, semen and uterine discharges for long period. We have successfully used a lytic *brucella* phage for therapy of Brucellosis in adult cattle. We also targeted the phage employing the live attenuated *Brucella abortus* strain 19 organisms to kill the virulent *Brucella* residing intracellularly in phagocytes in the body. The effect of both the therapies was monitored non-invasively employing *Brucella* RNA in blood plasma as a *Brucella* - specific biomarker. A single dose of the phage alone or the therapeutic vaccine (phage pulsed S-19) could substantially reduce and finally eliminate live *Brucella* in the body within 3 months as evident from diminished and ultimately non – detectable RNA characteristic of *Brucella abortus* (223 bp amplicon) in plasma by RT-PCR. Thus, phage has a potential to cure Brucellosis and abolish carrier state in cattle and RNA can serve as a specific biomarker of live *Brucella* for monitoring and assessment of the efficacy of the therapy.

Keywords: *Therapeutic Vaccine; Brucellosis; Brucella; Brucella Phage; Phage Therapy; Phage Targeting*

Introduction

Brucellosis caused by the facultative intracellular coccobacilli of genus *Brucella* is an important zoonotic disease. It causes abortion, delayed conception and temporary or permanent infertility in the affected animals resulting in huge economic loss to the livestock sector [1]. Brucellosis occurs worldwide but is controlled in developed countries. It is still prevalent in Middle East, Asia, Africa, South and Central America, the Mediterranean Basin and the Caribbean. Bovine Brucellosis is endemic in all states of India causing annual losses of Rs.5000 million [2,3].

Bovine Brucellosis is characterized by late term abortions and retention of placenta in females and orchitis and epididymitis in males, with excretion of organisms in semen, uterine discharges and in milk [4]. Once infected, the animal may become carrier and shed bacteria for long period [3]. Effective therapy for animals and human beings is still awaited and antibiotics are too expensive in most of the animal species [5]. Use of lytic bacteriophages to treat Brucellosis can be an alternative for control of the disease.

Bacteriophages are viruses that infect and multiply inside the bacteria. Lytic phages quickly reproduce within and lyse the bac-

teria and grow exponentially in the viable bacterial cells without damaging the normal flora. Bacteriophages that infect the *Brucella* species are called *Brucella* phages. Lytic bacteriophages can be safe alternative to antibiotics for treatment of bacterial diseases due to their self replicating and self-limiting nature [6]. We have shown that phage therapy can be successfully employed against Brucellosis in cattle [7]. The present study was planned to investigate the efficacy of *brucella* phage alone or *brucella* phage pulsed live *Brucella abortus* S-19 vaccine in clearance of *Brucella* organisms in cattle naturally infected with *Brucella abortus* employing a specific biomarker.

Materials and Methods

Brucella abortus strain 19 (vaccine strain)

The standard vaccine strain *Brucella abortus* strain 19, procured from the Biological Standardization Division, IVRI, Izatnagar was used in the present study. The culture was maintained on *Brucella* agar slants by serial sub-culturing every fortnight and storing the cultures at 4°C. The identity of *Brucella abortus* strain 19 was confirmed by staining (Gram's stain and *Brucella* differential staining - Stamp's modification of Ziehl Neelsen Method), microscopy, standard biochemical tests and PCR.

Brucella phage

A broad acting phage lytic to *Brucella* species isolated in our laboratory [8] was used for the present study. The phage stored as a crude, concentrated suspension in SM diluent, was revived by agar overlay technique as described by McDuff., *et al.* [9]. Tubes containing about 3 ml of melted NZCYM (Ameresco) soft agar i.e. NZCYM broth containing 0.75% Agar, tested overnight for sterility, were kept in a water bath adjusted to 47 - 48°C for 3-4 hours. About 0.8ml of log phase *Brucella abortus* S19 culture tested for purity and 0.6 ml of crude phage concentrate diluted to $1:10^2$ were added. The tubes were left at room temperature for about half an hour. The content of each tube was then overlaid on a separate 90 mm Petri plate containing hard sterile *Brucella* selective agar. All the plates were left at room temperature to allow soft agar to solidify and then incubated at 37°C aerobically for 2 - 3 days. The plates having plaque formation were confirmed for presence of phage by secondary streaking.

100µl of overnight grown *Brucella* strain 19 culture was added to 3 ml molten cooled semisolid NZCYM agar, mixed well and poured on NZCYM+ BSM agar plates. The plates were allowed to solidify at room temperature. The plaques were picked by platinum wire loop and streaked firstly as horizontal lines across the plate and later on as vertical lines dissecting the horizontal lines across the plate at 90°. The plates were then incubated at 37°C aerobically for 2-3 days. The clearing along the streak line indicated the presence of *brucella* phage. For further use the phage was eluted from the plates into sterile SM buffer.

Sterile SM buffer (5 ml) was poured over bacteriophage plates showing clearance around streak lines. The plaques along the line were disturbed using a platinum loop and the plates were kept in an incubator at 37°C up to 12 hours. The elutant was collected and centrifuged to discard any agar particle. Later on, the supernatant was filtered through 0.22 µm PVDF filter and preserved at 4°C.

Preparation of pure phage stock (Master lot)

The phage preparation obtained as mentioned above was amplified to 250 ml master lot using the liquid culture method as described by Rawat and Verma [10]. To about 250 ml NZCYM broth culture of *Brucella abortus* strain 19, purified phage obtained as described above was added aseptically in the phage-bacteria ratio of 1:50. The mixture was incubated at 37°C with intermittent shaking for a period until complete clearance of the turbidity could be observed indicating complete lysis of the organism. The phage-count of the preparation was evaluated by agar-overlay technique. The preparation was stored at 4°C.

Estimation of plaque forming unit (Pfu count)

Serial 10-fold dilutions of the purified phage stock up to 10^{-12} were prepared in sterile SM buffer. Equal quantity of each phage dilution was mixed in 3ml semisolid NZCYM agar at 47°C and seeded with the log phase culture of *Brucella abortus* strain 19. After mixing properly, the soft agar in each tube was plated on hard *Brucella* agar plates and left for solidifying. All the plates were then incubated at 37°C for 48 hours. The plaques produced were counted and phage count was determined. The phage titer was expressed in pfu/ml after multiplying with the dilution factor.

Pfu/ml= No. of plaques x Dilution factor/Volume of phage used.

Determination of multiplicity of infection (MOI) of the phage

The indicator strain *Brucella abortus* S19 was grown in about 100 ml NZCYM broth at 37°C (log phase). The bacterial cells were

harvested in fresh sterile NZCYM broth aseptically after centrifugation at 5000 rpm for 20 min. To approximately 10^8 cells/ml of exponentially grown *Brucella abortus* S19 resuspended in sterilized NZCYM broth medium and distributed in 6 separate tubes, the phage was added to make final phage: bacterial ratios of $1:10^4$, $1:5x10^3$, $1:10^3$, $1:5x10^2$, $1:10^2$ and 1:50, respectively and mixtures were incubated at 37°C. The phage - bacterial cell mixtures were examined visually for clearance of NZCYM broth medium and the total bacterial counts were determined at regular interval till complete clearance of the turbidity. The optimum phage: bacteria ratio that showed complete lysis of indicator strain *Brucella abortus* strain 19 within shortest period of time was considered as MOI of the phage for all future use.

Characterization of the phage

Determination of host range and heterogenicity test

The lytic activity of *brucella* phage was tested against *Brucella abortus* strain 99 and *Brucella melitensis* (procured from IVRI, Izatnagar) and several bacteria of heterologous species viz. *Staphylococcus aureus, Salmonella species, Escherichia coli* and *Pastuerella multocida.* 100 µl of overnight grown broth culture of test bacteria was added to 3ml molten cooled semisolid NZCYM agar, mixed well and poured on NZCYM + BSM agar plates. The plates were allowed to solidify at room temperature. The plaques suspected for *brucella* phage were picked up by platinum wire inoculation loop and streaked firstly as horizontal lines across the plates and later on as vertical lines dissecting the horizontal lines across the plate at 90°. The plates were then incubated at 37°C aerobically for 2 - 3 days.

Morphological characterization of the *brucella* phage

For studying the plaque morphology, the plaques were evaluated on the basis of their shape and size.

Preparation of phage suspension for therapy

Phage preparation was produced employing standardized optimum conditions as described below. The eluted phage preparation in SM diluent was purified using 0.22 µm PVDF filter before preparing the phage suspension for therapy. The phage count in the preparation was made to the required dose of 10^8 pfu/ml and stored at 4°C.

Preparation of phage based therapeutic vaccine

Vaccine strain and the phage

Organisms of *Brucella abortus* live attenuated strain 19 obtained from the vaccine Bruvax (Indian Immunologicals) was used for targeting the phage in this study. To 2 ml of the reconstituted *Brucella abortus* strain 19 vaccine (10^8 cells/ml) the phage was added to make the final phage: bacteria ratio equivalent to MOI (1:50). The mixture was incubated at 37°C and sterilized by filtration. The supernatant left after centrifugation was subjected to phage titer (pfu/ml) estimation. The therapeutic vaccine was inoculated into the brucellosis affected animals within 5 hours of preparation. This provides sufficient time for internalization of phage into the bacterial cell before lysing it.

Adjuvant

Sterilized 1 percent Aluminum Hydroxide gel suspension in saline was mixed aseptically with the test preparation in the ratio of 1:10 (final Aluminum concentration 0.1%) and incubated at 37°C for 24 hours and then stored at 4°C.

Sterility testing of phage preparation and the phage pulsed vaccine

Sterility tests on both the preparations were carried out before the commencement of immunization as recommended for the vaccines in the Indian Pharmacopoeia 2010 [11] Section 2.2.11. A loopful of the lysate was suspended in 5ml BHI broth as well as streaked on BHI and blood agar plates followed by incubation at 37°C. The broth and the plates were examined up to 48 hours for any microbial growth.

Ethical Committee approval

All the experimental protocols performed on laboratory animals and cattle were approved by the Institutional Animal Ethics Committee (IAEC) of GADVASU, Ludhiana. Animals were kept in IAEC approved facilities and received feed and water *ad libitum*.

Safety test of the phage preparation and the therapeutic vaccine in mice

Safety test was conducted on both the preparations by injecting 0.5 ml of test preparation through subcutaneous, intramuscular and intraperitoneal routes into each of the three mice in both the groups. The animals were observed for any untoward reactions or mortality till 7^{th} day of inoculation.

Treatment trials in cattle

Treatment trials of the two preparations were carried out at the University Dairy Farm, GADVASU, Ludhiana on Holstein Friesian

crossbred adult cattle which had a history of abortion and were Brucellosis positive by RBPT and PCR.

Experimental design

The Brucellosis positive adult cattle were divided into three groups viz; Group I (cattle treated with *brucella* phage preparation, n = 6); Group II (Cattle treated with *S19* vaccine pulsed with *brucella* phage, n = 6) and Group III (Brucellosis positive animals without any treatment, control animals, n = 3). Group I animals were inoculated with 2 ml of phage alone preparation through subcutaneous route whereas Group II animals received immunization with 2 ml of S19 vaccine pulsed with *brucella* phage through subcutaneous route. Group III animals which served as the control did not receive any kind of treatment.

Collection of blood

Blood samples were collected through jugular vein at 0, 30, 45, 60, 75 and 90 days post treatment for obtaining plasma for detection of nucleic acid of the *Brucella* organisms. Samples of plasma were stored at -80°C till further use.

Serological screening for brucellosis

Rose bengal plate agglutination test (RBPT)

Equal volumes (10 µl each) of RBPT colored antigen (Punjab Veterinary Vaccine Institute, Ludhiana) and serum from cattle were mixed on a clean glass slide with the help of a sterilized toothpick as per the method of Morgan., *et al.* [12]. The slide was observed for 4 min for the formation of clumps. Clumps formation was considered a positive test while the absence of clear clumps was considered a negative reaction.

Non invasive monitoring of treated animals employing a biomarker

Detection of nucleic acid by reverse transcriptase polymerase chain reaction (RT-PCR)

RNA isolation

Total RNA was isolated from the plasma samples using RNA isolation kit (TRI reagent BD; Sigma Aldrich). Plasma sample (250µl) was collected in DEPC treated 2 ml Eppendorf tube containing 750 µl TRI reagent BD. Tubes were mixed gently by vortexing and incubated at room temperature for 15 minutes. 200 µl of chloroform was added into each tube. The tubes were mixed gently by vortexing and incubated at -20°C for 15 minutes. The tubes were centrifuged at 11,000 rpm for 15 minutes at 4ºC. The upper aqueous phase containing RNA was transferred into fresh, clean, sterile, autoclaved Eppendorf tubes. To the upper aqueous phase, equal volume of RNase-free isopropanol was added. The tubes were mixed gently by inversion and kept at room temperature for 30 - 45 minutes to allow precipitation of RNA. The contents were centrifuged at 11,000 rpm for 15 minutes at 4ºC. The supernatant was discarded by gentle inversion. The RNA pellet was washed in 500 µl of 75% ethanol. 500 µl of 75% ethanol was added and the Eppendorf tubes were centrifuged at 7500 rpm for 10 minutes at 4ºC. Finally, the RNA pellet was air dried after inverting on blotting paper so that last trace of ethanol was evaporated. 10 µl of nucleases free water was added and the Eppendorf tubes were stored at 4ºC for 2 - 3 hours to dissolve the RNA.

Quantification of RNA

The optical density of the nucleic acid was measured in an ultraviolet ray Nanodrop spectrophotometer. For quantification of the amount of RNA, readings were taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows the calculation of the concentration of nucleic acid in the sample.

Pure preparations of RNA with OD260/OD280 ranging between 2.0 - 2.2 were selected for further analysis. The concentration of total RNA varied between 600 to 2500 ng/µL in different samples. The amount of total RNA used for cDNA synthesis was 2 µg for each sample.

Positive control used for RT-PCR

The RNA isolated from the *Brucella abortus* strain 19 organisms from the live attenuated vaccine Bruvax (Indian Immunologicals) was used as a positive control for the RT-PCR detection of *Brucella abortus*.

First strand cDNA synthesis

Total RNA extracted was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as follows:

After thawing, all the components of the kit were mixed properly and were kept on ice. In the first step, 2 - 5 µl of total RNA extracted from each sample was taken into separate sterilized PCR tubes (200 µl) and nuclease free water was added into each tube, making a total volume of 10µl (Table 1).

RNA	2 - 5 µl (2 µg)
Nuclease free water	Up to 10 **µl total volume**
Total volume	10 **µl**

Table 1: RNA preparation.

The following reaction mixture was prepared for reverse transcription (Table 2).

10x RT Buffer	2 µl
25 mM dNTP Mix	0.8 µl
10x Random Primer	2 µl
RT	1 µl
NFW	4.2 µl
Total volume	10 µl

Table 2: Reaction mixture for reverse transcription.

10 µl of the above mixture was added into the PCR tubes containing RNA and Nuclease Free Water (NFW) making a total volume of 20 µl after proper mixing and brief centrifugation. The mixture was set in PCR for reverse transcription of mRNA into cDNA (Table 2).

Expression profiling by RT PCR

The RT-PCR assay for the amplification of the gene encoding 31 kDa *B. abortus* antigen was carried out using *Brucella* genus specific primers B4/B5 [13]. These were got synthesized from Promega as per the sequences given in the table 3.

Primers	Sequence	Size of amplified product	Reference
B4 F	5'-TGG CTC GGT TGC CAA TAT CAA-3'	223 bp	Baily., *et al.* (1992)
B5 R	5'-CGC GCT TGC CTT TCA GGT CTG-3'		

Table 3: Sequence of primers used for detection of *B. abortus.*

RT-PCR for detection of *Brucella* specific nucleic acid

In this study, cDNA prepared from the RNA extracted from plasma samples was subjected to PCR amplification of B4/B5 gene. The reaction mixture (Table 4) was prepared using gene specific primers.

Master mix component	Volume per reaction (µl)
Master mix (Promega)	12.5
Primer-B4 (forward) (10p Moles)	0.5
Primer-B5 (reverse) (10p Moles)	0.5
$MgCl_2$ (25mM)	0.5
Template cDNA	8
RNase free water	3
Total volume	**25.0**

Table 4: Composition of the PCR reaction mixture.

Above reaction mixture was mixed properly and briefly spun. PCR tubes were loaded to the thermocycler with following cycling conditions (Table 5).

Temperature	Time	No. of cycles	Remarks
25°C	10 min	1	Incubation
37°C	120 min	1	Reverse transcription
85°C	5 min	1	Stopping the reaction

Table 5: PCR conditions followed during first strand synthesis of cDNA.

PCR products obtained were stored at -20°C till further use.

Agarose gel electrophoresis

Agarose (0.6g) was dissolved in 30 ml of 0.5X TBE, heated to melt and allowed to cool to 50°C. Then 4 µl of Ethidium Bromide (10 mg/ml stock at final concentration of 0.5 µg/ml) was added and mixed thoroughly. This mixture was poured into a gel-casting tray fit with comb and allowed to solidify. Once the gel got solidified, a few ml of TBE (0.5X) was added. The comb was removed carefully and the gel was immersed in the electrophoresis tank containing 0.5X TBE buffer. Samples were loaded into the wells. Electrophoresis was carried out at 80 - 100 volts/cm. The bands were visualized under UV- illumination (Bio-Rad, USA). The amplicon size and concentrations were determined by comparing with the Gene Ruler 100 bp plus DNA ladder which was run along with the samples.

Results

The present study was designed to explore the efficacy of *brucella* phage in the therapy of cattle naturally infected with *Brucella abortus*. The study also aimed at development of a new therapeutic vaccine (phage pulsed vaccine; *Brucella abortus* strain 19 vaccine pulsed with *brucella* phage) and evaluating its efficacy in clearance of *Brucella* organisms in cattle.

The following strategy was employed:

- Generation of *brucella* phage alone preparation for therapy and *Brucella abortus* strain 19 vaccine pulsed with *brucella* phage for use as a therapeutic vaccine.
- Therapeutic trials of the two preparations in cattle naturally affected with brucellosis.
- Employing a molecular biomarker to monitor the efficacy of the two preparations in clearance of the *Brucella* organisms.

Bacterial strain

Morphological characteristics of the colonies - like smooth, glistening, translucent and pinpoint colonies, appearing after 3 - 5 days of incubation in a micro-aerophilic environment indicated *Brucella* organisms. The organisms were found to be Gram negative coccobacillary rods whereas by modified Ziehl Neelsen staining (MZN) they appeared to be red coccobacilli against a blue background. The organisms were found to be positive for catalase, H_2S, oxidase and urease tests. The indicator strain S-19 culture was maintained on *Brucella* agar slants by serial sub-culturing fortnightly and storing the cultures at 4°C.

Brucella phage

A phage lytic to *Brucella* organisms isolated in our laboratory earlier was used for the present study. *Brucella abortus* strain 19 organisms was used for the revival of the stored phage. The plates having plaque formation (Figure 1) were confirmed for the presence of phage by secondary streaking (Figure 2).

The *brucella* phage lysed *Brucella abortus* strain 19, *Brucella abortus* strain 99 and *Brucella melitensis* (procured from IVRI, Izatnagar) organisms but did not lyse any of the heterologous species tested viz. *Staphylococcus aureus, Salmonella species, Escherichia coli* and *Pasteurella multocida.*

Figure 1: Circular plaques caused by lysis of *Brucella.*

Figure 2: Lytic zones created after secondary streaking of phage on *Brucella* lawn.

The observed plaques were circular. The *brucella* phage belongs to the order *Caudovirales* and family *Siphoviridae.*

Multiplicity of infection (MOI)

The optimum phage-bacteria ratio to achieve maximum lysis of the indicator strain (*Brucella abortus* strain 19) within the shortest period of incubation was 1:50. The results are summarized in table 6.

Pfu/ml of the phage calculated by several dilutions of the phage in SM buffer followed by plating (agar overlay technique) was 2.0 x

Phage: Bacteria ratio	Total viable count (CFU / ml) in Brucella agar plates			
	30 min	60 min	90 min	180 min
1: 10^4	3.0 x 10^8	5.5 x 10^8	8.0 x 10^7	2.2 x 10^6
1:5 x 10^3	2.0 x 10^8	1.0 x 10^8	9.4 x 10^7	6.0 x 10^6
1: 10^3	1.5 x 10^8	6.2 x 10^8	3.0 x 10^7	9.0 x 10^6
1:5 x 10^2	1.2 x 10^8	8.1 x 10^7	5.9 x 10^6	1.1 x 10^6
1:10^2	1.2 x 10^8	7.5 x 10^7	4.0 x 10^6	8.0 x 10^5
1:50	1.0 x 10^8	6.0 x 10^7	2.0 x 10^6	4.8 x 10^5
Control	1.0 x 10^8	4.0 x 10^8	1.2 x 10^8	2.5 x 10^9

Table 6: MOI of the *brucella* phage.

10^8 pfu/ml. No reduction in count was observed during the entire period of investigation.

"Phage alone" preparation for therapy

The phage count of the preparation was made to a dose of 2 x 10^8 pfu/ml and sterile aluminum hydroxide gel suspension in saline in ratio of 1:10 was added to the preparation.

Therapeutic vaccine (*Brucella abortus* strain 19 pulsed with *brucella* phage)

The phage alone and phage pulsed vaccine preparations were found to be bacteriologically sterile and free from any fungal contamination when tested on BHI and blood agar plates. Safety tests of the preparations were conducted on mice as per the protocol recommended in the Indian Pharmacopoeia 2010. The mice inoculated through the three routes of administration did not reveal any untoward reaction or death during an observation period of 7 days. The preparations were found to be safe in mice.

Monitoring the effect of phage therapy employing a biomarker

For studying the efficacy of phage in clearance of *Brucella* organisms, Reverse Transcriptase PCR assay was employed to monitor a nucleic acid biomarker. RNA was isolated from the plasma samples using RNA isolation kit. Pure preparations of RNA with OD 260/OD 280 ratio ranging between 2.0 - 2.2 were selected. The concentration of total RNA varied between 600 to 2500 ng/µL in different samples. The amount of total RNA used for cDNA synthesis was 2µg for each sample.

The complementary DNA (cDNA) synthesized from RNA was amplified for the gene encoding 31 kDa *Brucella abortus* antigen (*bcsp 31*) using B4/B5 primers. At 0 day, the 31 kDa *bcsp* PCR assay produced amplicon at 223 bp when applied to all the samples from infected animals used in the trial. This also confirmed the results obtained from serodiagnostic test for the brucellosis positive status of the animal.

After administration of *brucella* phage alone and the new therapeutic vaccine in animals, detection of RNA in the plasma was carried out fortnightly for three months. Plasma samples collected from the treated animals on 30, 45, 60, 75- and 90-days post treatment were subjected to RT- PCR (Figure 3). There was a marked reduction in the thickness of the band produced at amplicon size of 223 bp from 0 to 90-day post treatment in samples from group I (*Brucellosis* positive adult cattle treated with *brucella* phage preparation) and group II (*Brucellosis* positive cattle treated with *S19* vaccine pulsed with *brucella* phage). The faint band or absence of band at 90 day suggested reduced load to complete elimination of *Brucella* organisms in the body. There were no changes till 90 day compared to 0 day in amplicons produced from group III samples (Brucellosis affected animals which received no treatment). The samples from *Brucella abortus* strain 19 vaccinated animals which did not receive any kind of phage treatment showed positive result on RT-PCR examination indicative of viable S-19 organisms. However, the sample of a control animal (one-month old brucellosis free calf which was not vaccinated against *Brucella abortus* and received no treatment with phage or phage pulsed vaccine), gave no bands at the required amplicon size.

Discussion and Conclusion

The phage pulsed therapeutic vaccine was inoculated into the brucellosis affected animals within 5 hours of preparation. It provided sufficient time for internalization of phage into the bacterial cell without lysing it. Gupta and Saxena [14] monitored phage mediated lysis of *Brucella abortus* strain 19 by release of ATP and observed that after 5 hours of incubation of phage-bacteria suspension, there was considerable increase in extracellular ATP in the phage lysed bacteria compared to bacterial suspension alone.

In our study, the fainter bands obtained at 223 bp from the therapeutic vaccine treated animals on 90 days post treatment, may be due to the reduced level of the *Brucella* S-19 organisms. The phage

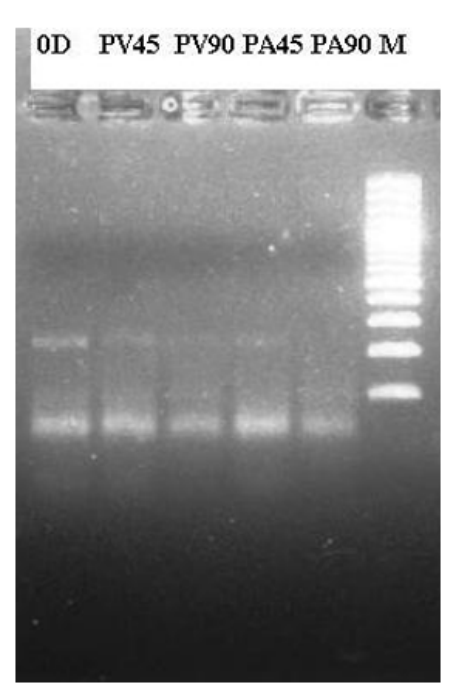

Figure 3: RTPCR of RNA of Brucellosis affected cows treated with phage or phage pulsed S19.
Lanes (L to R): L1 = 0 day, L2 = phage pulsed vaccine (PV) 45 day, L3 = PV 90 day, L4 = phage alone (PA) 45 day, L5 = PA 90 day, M = marker.

in the therapeutic vaccine preparation could have possibly lysed the live S-19 bacteria present in the vaccine after reaching the site of predilection of *Brucella* organisms in the body. In control group, the bands remained without any change upto 90 days indicating the continued presence of live *Brucella abortus* organisms.

The *Brucella abortus* amplified gene detected in brucellosis free vaccinated animals, could probably be due to the live *Brucella abortus* organisms in the strain 19 vaccine which remained viable after eliciting the immune response in the animal. The possibility remains that such animals may act as carriers by shedding the organisms in the secretions or excretions. The new therapeutic vaccine has the advantage of eliminating the live *Brucella abortus* S-19 organisms also after the induction of immune response by the vaccine.

In principle, the presence of nucleic acid in bacterial cells might be a useful indicator of viability [15]. DNA targeted PCR is incapable of determining viability as DNA is having high stability and is also demonstrated to persist in a PCR detectable form in culture negative environmental and clinical samples [16,17]. According to Masters., *et al.* [18], the correlation between cell viability and detection of DNA was poor, as DNA was shown to be persistent in actively killed cells for significant periods of time.

The phosphodiester bonds of RNA are more susceptible to hydrolysis than those of DNA because of the presence of 2'-OH group of ribose sugar. Therefore, it is more labile and susceptible to degradation [15]. Due to the short half life and liability of RNA, it has been considered as a plausible indicator of viability and diagnostic target for microbial infections [19]. RT-PCR has historically been the amplification method of choice when analyzing RNA, mainly as PCR became established as a key technique for many DNA-based measurements. In a study conducted by Ling and Nielsen [20], PCR method targeting *bcsp 31* was found to be more sensitive than that for outer membrane protein 2 (*omp 2*) and 16S rRNA. Mukherjee., *et al.* [21] opined that 16S rRNA gene PCR used for detection of bovine blood samples was insensitive. The result is consistent with that reported by Elfaki [22]. Al-Ajlan., *et al.* [23] also reported that B4/B5 amplification was most sensitive as it could amplify DNA extracted as low as 25 and 100 cfu/ml in 1 ml water and blood, respectively [24].

The effect of *brucella* phage on the course of infection with *Brucella abortus* was examined by Morris (1980). He reported that *Tbilisi* phage reduced the level of infection with phage sensitive but not with phage resistant *B. abortus* in bovine monocyte cell cultures.

Prajapati., *et al.* (2014) investigated the preventive and therapeutic potential of *brucella* phage in experimentally infected mice with *Brucella abortus* strain 544 and concluded that it is a promising alternative for reducing *Brucella* colonization in mice.

The current investigation is perhaps the first systematic attempt whereby efficacy of *brucella* phage alone and *brucella* phage pulsed vaccine preparations was evaluated in cattle non-invasively. We have not come across any published reports documenting the detection of ribosomal RNA characteristic of the bacteria particu-

larly in the large animal model for monitoring the effect of phage therapy. Our present study may possibly be the first to demonstrate the effect of phage therapy on bacterial load monitored by RT-PCR of the RNA of the bacteria in blood plasma in case of bovine brucellosis. Although our results with biomarker-based monitoring of phage therapy are quite encouraging, long term studies on larger number of animals are required to get a conclusive picture.

Author Contributions

AM carried out the experimental work on phage therapy and therapeutic vaccine trial. HMS conceived the idea and designed the study, analyzed and interpreted the data and drafted the article. Both the authors have approved the final article.

Conflict of Interest

The authors declare no conflict of interest.

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