



## Effects of Bacteriophage Therapy on Host Immune Responses in Brucellosis Affected Cattle

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### Abstract

We studied the effect of phage therapy on immune responses in Brucellosis affected cattle. There was a very significant ( $p < 0.01$ ) increase in total serum proteins from 0D to 45D, 60D and 75D and from 30D to 45D and a decrease from 45D to 90D, respectively. There was a significant ( $p < 0.05$ ) increase in total serum proteins from 0D to 30D and 90D and a decrease from 45D to 75D. Serum globulin level increased very significantly ( $p < 0.01$ ) from 0D to 45D and 60D. The increase in serum globulin level from 30D to 45D and decrease from 45D to 90D were significant ( $p < 0.05$ ). There was a very significant ( $p < 0.01$ ) rise in anti-Brucella antibody titers estimated by Standard Tube Agglutination Test (STAT titers) from 0D to 45D and 60D along with a significant ( $p < 0.05$ ) decline from 45D to 90D. There was a very significant ( $p < 0.01$ ) rise in MAT titers from 0D to 45D and 60D along with a decline from 45D to 90D. The rise in titer from 30D to 45D was significant ( $p < 0.05$ ). There was a very significant ( $p < 0.01$ ) rise in IHA titers from 0D to 45D and 60D and a significant ( $p < 0.05$ ) rise from 0D to 75D. There was a very significant ( $p < 0.01$ ) decline in ELISA titers from 30D to 60D, 75D and 90D, respectively. The decline in titers from 0D to 60D and 75D and from 45D to 60D and 75D was significant ( $p < 0.05$ ). There was a very significant ( $p < 0.01$ ) decrease in the levels of lymphocytes from 0D to 30D followed by increase from 30D to 45D, 60D and 75D. The increase from 30D to 90D was very significant ( $p < 0.01$ ). However, Levels of leukocytes, monocytes and neutrophils did not differ significantly among themselves during the period of 90 days after treatment with the phage. This suggests that phage therapy in Brucellosis stimulates immune responses in cattle initially which decline slowly after 45 days.

**Keywords:** Phage Therapy; Brucellosis; Antibody Titers; Leukocytes; Lymphocytes; Neutrophils

### Introduction

Brucellosis is a major bacterial zoonosis of global importance. About 500,000 cases of human brucellosis are estimated to occur worldwide every year.

Bovine brucellosis is endemic in all states of India. In India, the occurrence of brucellosis is to the extent of 10% in the marginal herds and 50% in organized farms, and the socio-economic impact of the disease was estimated to run over Rs. 500 crores annually. In Punjab, overall 17.7% prevalence of brucellosis was reported in cattle and buffaloes [1,2]. It causes heavy economic loss to the animal industry through delayed conception, late-term abortions and retention of placenta and temporary or permanent infertility [3] 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 continue to shed bacteria and remains a source of infection to others for long period [2]. Sometimes the vaccinated animals may also suffer from Brucellosis due to inadequate

immunity induced by the vaccine [5]. Phage therapy may be effective in chronic carriers infected with antibiotic resistant bacteria. However, little data are available in the published literature on the effects of phage therapy on host immune response to natural infection of Brucellosis in cattle. The present study was, therefore, undertaken to explore this aspect of phage therapy of bovine brucellosis.

### Materials and Methods

#### Ethical approval

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

#### Infected cattle

Six naturally infected brucellosis positive adult cattle, which had been vaccinated during calf hood with *B. abortus*\_strain 19 vaccine

(Bruvax; Indian Immunologicals) maintained at the University Dairy Farm were included in the study.

### Preparation of phage for therapy

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

### Sterility testing of phage preparation

Sterility test was carried out as per the Indian Pharmacopoeia Section 2.2.11. A loopful of the phage preparation 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 upto 48 hours for any microbial growth.

### Safety test of phage in mice

Safety test was conducted by injecting 0.5 ml of phage preparation through subcutaneous, intramuscular and intraperitoneal route into each of the three mice. The mice were observed for any untoward reactions or mortality till 7<sup>th</sup> day of inoculation.

### Treatment trials in cattle

Treatment trials of the phage preparation were carried out on naturally Brucellosis infected Holstein Friesian crossbred adult cattle at the University Dairy Farm, GADVASU, Ludhiana. Six Brucellosis positive adult cattle were treated with 2 ml of 10<sup>8</sup> pfu/ml brucellaphage preparation through subcutaneous route.

### Collection of blood

Blood samples were collected from cattle through jugular vein at 0, 30, 45, 60, 75 and 90 days post treatment. Anti-coagulated blood was collected for separating leukocytes while sera were separated from clotted blood and stored at -20°C until further use for studying the immune response of the treated animals.

### Analysis of immune responses

#### Estimation of total protein levels in serum samples

The total protein content in serum samples of phage treated cattle at 0, 30, 45, 60, 75 and 90 days post treatment were determined by using Vitros TP slides and the Vitros DTSC (Johnson and Johnson Company) instrument.

#### Estimation of total globulin levels in serum samples

Total globulin levels in the serum samples of treated cattle at 0, 30, 45, 60, 75 and 90 days post treatment were estimated by subtracting albumin content from the total protein level determined

by using Vitros TP slides, Vitros ALB DT slides and the Vitros DTSC (Johnson and Johnson Company) instrument.

#### Estimation of antibody titers

Titers of anti-Brucella antibodies in the serum samples of phage treated cattle at 0, 30, 45, 60, 75 and 90 days post treatment were estimated by Standard Tube Agglutination Test (STAT), Microtiter plate Agglutination Test (MAT), Indirect Hemagglutination Assay (IHA) and Enzyme-Linked Immunosorbent Assay (ELISA). The methods followed have been described earlier [5].

#### Estimation of total leukocyte counts (TLC) in the blood of treated cattle

Levels of total leukocytes in blood samples of phage treated Brucellosis affected cattle at 0, 30, 45, 60, 75 and 90 days post treatment were determined. Approximately 20µl of blood from treated cattle was mixed with 300µl of WBC diluting fluid (BTL Research Lab, India) and kept for 10 minutes undisturbed. One drop of the diluted blood was loaded into Neubauer's counting slide and a cover slip was put over it. The number of cells in the WBC counting chamber was counted under a microscope.

#### Estimation of differential leukocyte counts (DLC) in the blood of treated cattle

Levels of leukocyte subsets in blood samples of phage treated Brucellosis affected cattle were determined at 0, 30, 45, 60, 75 and 90 days post treatment. Blood smear from treated cattle was prepared on a clean microscopic slide and the slide was air dried. Fixation was done by immersing the slide in methanol for around 5 minutes. The slide was flooded with Giemsa stain and kept for 10 minutes. Afterwards it was washed in running water and the slide was kept for drying. The slide was observed under a microscope and the total numbers of cells were counted upto 100 cells.

#### Statistical analysis of data

Data were statistically analyzed by analysis of variance and t-test.

### Results and Discussion

Bacteriophages are viruses that infect and multiply inside the bacteria. Bacteriophages that infect the *Brucella* species are called Brucellaphages. Use of lytic bacteriophages to treat Brucellosis can be a cheap and effective alternative to antibiotics for control of the disease. Lytic phages grow in the viable bacterial cells, reproduce within and lyse the bacteria without damaging the normal flora [6]. The self-replicating and self-limiting nature of lytic bacteriophages make them a safe alternative to antibiotics for treatment of bacterial diseases of animals [6]. Our present study was undertaken

to investigate the effect of brucellaphage therapy on immune responses in cattle naturally infected with *Brucella abortus*.

A broad acting phage lytic to *Brucella* organisms isolated in our laboratory was used for the present study. *Brucella abortus* strain 19 organism was used for the revival and propagation of phage. The plates having plaque formation were confirmed for the presence of phage by secondary streaking. The brucellaphage lysed *Brucella abortus* strain 19, *Brucella abortus* strain 99 and *Brucella melitensis* (procured from IVRI, Izatnagar) but did not lyse any of the heterologous species tested viz. *Staphylococcus aureus*, *Salmonella* species, *Escherichia coli* and *Pasteurella multocida*.

The total serum protein concentrations (g/dl) in phage treated cattle suffering from Brucellosis (treated cattle) increased from 5.05 to 8.15 in 45 days and then slowly declined to 6.39 by 90 days post treatment (Table 1 and figure 1). The increase in total serum proteins from 0D to 45D, 60D and 75D and from 30D to 45D followed by a decrease from 45D to 90D, respectively was very significant ( $p < 0.01$ ). The increase in total serum proteins from 0D to 30D and 90D followed by a decrease from 45D to 75D was significant ( $p < 0.05$ ).

The serum globulin levels (g/dl) in treated cattle rose from 2.1 to 4.86 in one and a half month and thereafter gradually declined to 3.06 by 3 months post treatment (Table 2 and figure 1). The increase in serum globulin level from 0D to 45D and 60D was very significant ( $p < 0.01$ ). The increase in serum globulin from 30D to 45D and decrease from 45D to 90D were significant ( $p < 0.05$ ).

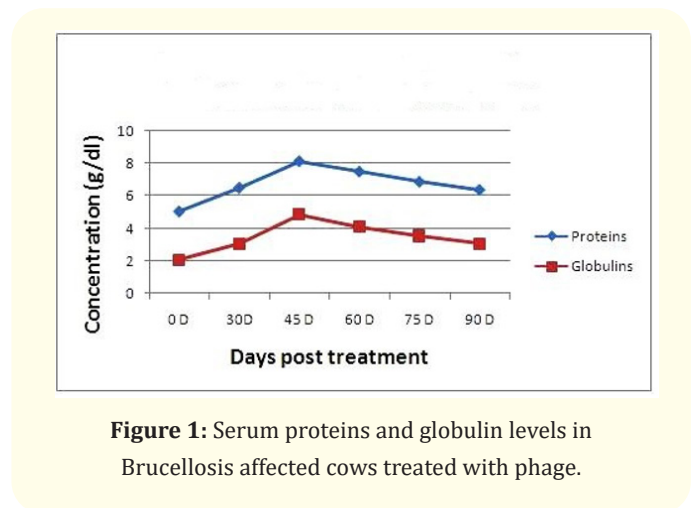
Titers of anti-Brucella antibodies as determined by STAT (STAT titers) increased gradually from  $1.953 \pm 0.29$  to  $2.755 \pm 0.12$  in one and a half month and thereafter slowly declined to  $2.153 \pm 0.44$  by 90<sup>th</sup> day post therapy (Table 3 and figure 2). The rise in STAT titers from 0D to 45D and 60D was very significant ( $p < 0.01$ ) The decline from 45D to 90D was significant ( $p < 0.05$ ). MAT titers increased gradually from  $1.802 \pm 0.24$  to  $3.759 \pm 0.44$  by 45 day and then declined slowly to  $2.404 \pm 0.80$  three months post treatment (Table 4 and figure 2). The rise in MAT titers from 0D to 45D and 60D, along with decline from 45D to 90D was very significant ( $p < 0.01$ ). The rise in titer from 30D to 45D was significant ( $p < 0.05$ ). IHA titers (Table 5 and figure 2) increased from  $2.053 \pm 0.31$  to  $3.859 \pm 0.36$  by 45 day along with a gradual decline to  $2.856 \pm 0.94$  after 3 months of therapy. The rise in IHA titers from 0D to 45D and 60D was very significant ( $p < 0.01$ ) and the rise from 0D to 75D was significant ( $p < 0.05$ ). ELISA titers rose from  $1.122 \pm 0.44$  to  $1.361 \pm 0.05$  at one month along with a gradual decline to  $0.803 \pm 0.17$  at 90 day (Table 6 and figure 2). The decline in ELISA titers from 30D

Sl. No.	0 D	30D	45 D	60 D	75 D	90 D
1	5.6	7.3	7.6	7.5	6.6	6.6
2	5.8	6.7	6.7	6.8	6.2	5.6
3	6.0	6.9	9.0	8.0	7.1	6.7
4	3.9	6.2	9.3	8.1	7.7	7.1
5	4.0	5.8	8.2	7.5	6.7	5.8
6	5.0	6.2	8.1	7.2	7.1	6.6
Mean $\pm$ SD	$5.05 \pm 0.91$	$6.51 \pm 0.54$	$8.15 \pm 0.94$	$7.51 \pm 0.48$	$6.89 \pm 0.51$	$6.39 \pm 0.57$

**Table 1:** Total serum protein levels in Brucellosis affected cattle after phage therapy.

Sl. No.	0 D	30D	45 D	60 D	75 D	90 D
1	2.1	2.3	4.7	4.4	4.0	3.8
2	2.4	2.8	3.7	3.7	3.0	2.6
3	3.0	3.1	6.6	4.5	3.8	3.8
4	2.4	4.9	6.0	5.0	4.0	3.6
5	1.0	1.7	4.4	3.5	3.2	2.0
6	2.8	3.6	3.8	3.7	3.3	2.6
Mean $\pm$ SD	$2.1 \pm 0.7$	$3.06 \pm 1.01$	$4.86 \pm 1.18$	$4.13 \pm 0.58$	$3.55 \pm 0.43$	$3.06 \pm 0.76$

**Table 2:** Serum globulin levels in Brucellosis affected cattle after phage therapy.



**Figure 1:** Serum proteins and globulin levels in Brucellosis affected cows treated with phage.

to 60D, 75D, and 90D, respectively was very significant ( $p < 0.01$ ). The decline in titers from 0D to 60D and 75D and from 45D to 60D and 75D was significant ( $p < 0.05$ ).

Total leukocyte count in the peripheral blood of Brucellosis affected cattle increased from 5766.66 to 8150 by one month followed by a decline to 6883.33 till 60 day and then gradual increase

Sl. No.	0 D	30D	45 D	60 D	75 D	90 D
1	2.204	2.505	2.806	2.806	2.806	2.806
2	1.602	2.204	2.806	2.806	1.903	1.903
3	1.602	2.505	2.806	2.204	1.903	1.602
4	2.204	2.505	2.806	2.806	2.806	2.204
5	1.903	2.204	2.505	2.505	2.505	2.505
6	2.204	2.505	2.806	2.505	2.204	1.903
Mean ± SD	1.953 ± 0.29	2.404 ± 0.15	2.755 ± 0.12	2.605 ± 0.24	2.354 ± 0.41	2.153 ± 0.44

**Table 3:** Antibody titers by standard tube agglutination test (STAT) in brucellosis affected cattle after treatment with phage.

Sl. No.	0 D	30D	45 D	60 D	75 D	90 D
1	1.602	2.204	3.408	3.408	3.408	3.408
2	1.903	3.107	3.709	3.709	1.903	1.903
3	1.602	1.903	4.311	3.408	2.806	2.204
4	1.602	1.903	3.408	3.408	3.408	1.903
5	1.903	2.204	3.408	3.408	3.408	3.408
6	2.204	3.709	4.311	2.204	1.903	1.602
Mean ± SD	1.802 ± 0.24	2.505 ± 0.73	3.759 ± 0.44	3.257 ± 0.52	2.806 ± 0.73	2.404 ± 0.80

**Table 4:** Antibody titers by microtitre plate agglutination test (MAT) in brucellosis affected cattle after treatment with phage.

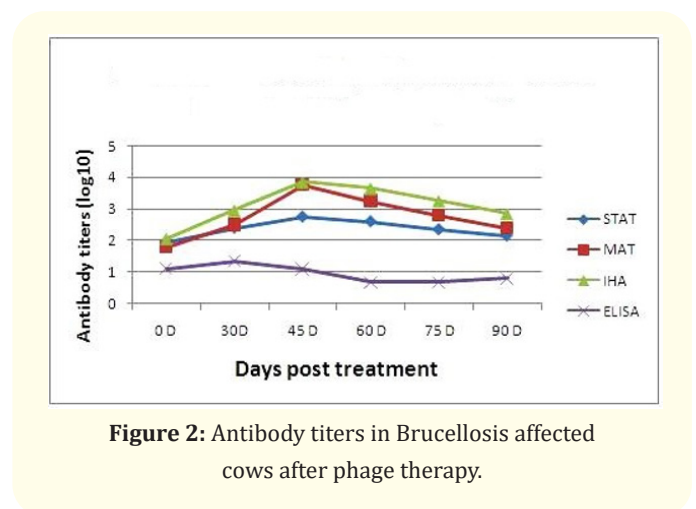
Sl.No.	0 D	30D	45 D	60 D	75 D	90 D
1	2.505	3.107	3.709	3.709	3.709	3.709
2	1.903	3.107	3.408	2.505	2.204	1.903
3	1.602	1.903	3.709	3.408	2.505	2.204
4	1.903	2.505	4.311	4.311	4.311	3.709
5	2.204	3.408	3.709	3.709	3.709	3.709
6	2.204	3.709	4.311	4.311	3.107	1.903
Mean ± SD	2.053 ± 0.31	2.956 ± 0.65	3.859 ± 0.36	3.658 ± 0.67	3.257 ± 0.80	2.856 ± 0.94

**Table 5:** Antibody titers by indirect haemagglutination test (IHA) test in brucellosis affected cattle treated with phage.

to 8183.33 by 3 months after initiation of phage therapy (Table 7 and figure 3). Blood lymphocyte levels (percentage) reduced considerably from 54.5 to 35.33 by 1 month and then rose to 60 by 45 day, followed by slight decline reaching 54.83 by 90 day post treatment (Table 8 and figure 4). Monocytes increased from 2.83 to 3.16 by 30 day and then declined to 1.66 by 45 day, maintained similar level till 75 day and then reached 2.16 by day 90 (Table

Sl. No.	0 D	30D	45 D	60 D	75 D	90 D
1	1.453	1.434	1.331	0.784	0.754	1.100
2	0.610	1.292	1.245	0.704	0.500	0.756
3	1.293	1.422	1.176	0.671	0.753	0.780
4	0.496	1.303	0.691	0.511	0.574	0.562
5	1.408	1.347	1.001	0.685	0.709	0.788
6	1.473	1.367	1.174	0.733	0.822	0.832
Mean ± SD	1.122 ± 0.44	1.361 ± 0.05	1.103 ± 0.20	0.681 ± 0.09	0.685 ± 0.12	0.803 ± 0.17

**Table 6:** Antibody titers (OD values) by ELISA in Brucellosis affected cattle treated with phage.



**Figure 2:** Antibody titers in Brucellosis affected cows after phage therapy.

Sl. No.	0 D	30D	45 D	60 D	75 D	90 D
1	5900	7500	5900	6100	5200	9200
2	4800	9200	8200	5200	6800	8100
3	6200	10200	7800	9800	7600	10200
4	6900	6500	5900	5800	9900	6500
5	5700	8300	9400	6900	8400	7300
6	5100	7200	8500	7500	8900	7800
Mean ± SD	5766.66 ± 758.06	8150 ± 1369.30	7616.66 ± 1430.26	6883.33 ± 1643.67	7800 ± 1660.12	8183.33 ± 1331.79

**Table 7:** Total blood leukocyte count (TLC) in Brucellosis affected animals treated with phage.

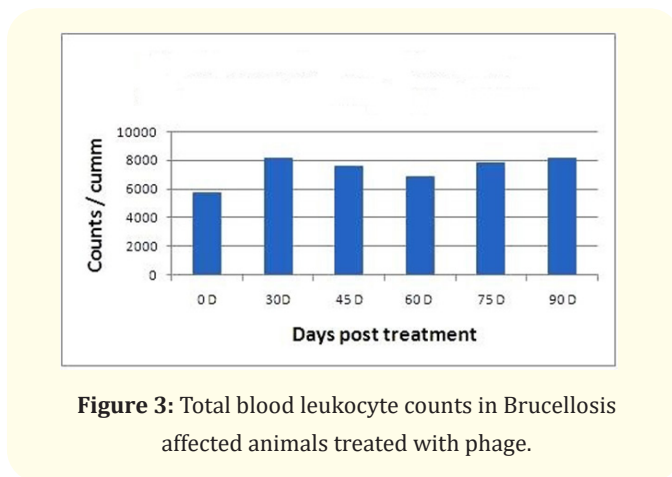
9 and figure 4). Neutrophil counts did not vary considerably from 39.83 at 0 day to 38.33 at day 75 and 40.83 at day 90 (Table 10 and figure 4). The decrease in the levels of lymphocytes from 0D to 30D followed by increase from 30D to 45D, 60D, 75D and 90D was very

Sl. No.	0 D	30D	45 D	60 D	75 D	90 D
1	66	50	55	56	50	51
2	54	30	62	58	58	50
3	55	24	64	65	49	62
4	50	44	53	63	65	55
5	46	34	68	60	60	53
6	56	30	58	57	58	58
Mean ± SD	54.5 ± 6.74	35.33 ± 9.77	60 ± 5.69	59.83 ± 3.54	56.66 ± 6.12	54.83 ± 4.53

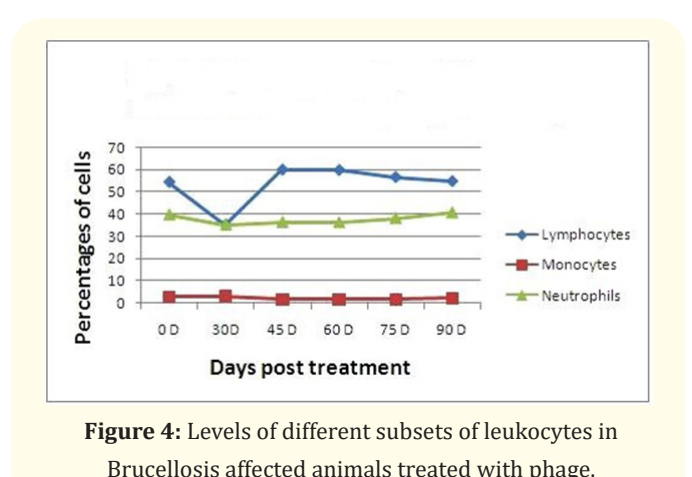
**Table 8:** Blood lymphocyte counts in Brucellosis affected animals treated with phage.

Sl. No.	0 D	30D	45 D	60 D	75 D	90 D
1	31	50	42	40	48	44
2	39	30	33	36	38	44
3	36	24	33	31	46	33
4	43	44	44	33	30	42
5	50	34	28	38	32	43
6	40	30	39	40	36	39
Mean ± SD	39.83 ± 6.43	35.33 ± 9.77	36.5 ± 6.15	36.33 ± 3.72	38.33 ± 7.31	40.83 ± 4.26

**Table 10:** Blood neutrophil counts in brucellosis affected animals treated with phage.



**Figure 3:** Total blood leukocyte counts in Brucellosis affected animals treated with phage.



**Figure 4:** Levels of different subsets of leukocytes in Brucellosis affected animals treated with phage.

Sl. No.	0 D	30D	45 D	60 D	75 D	90 D
1	1	3	2	2	1	3
2	4	4	3	4	2	3
3	4	2	1	1	3	2
4	4	4	1	1	2	1
5	1	3	2	1	4	2
6	3	3	1	1	2	2
Mean ± SD	2.83 ± 1.47	3.16 ± 0.75	1.66 ± 0.81	1.66 ± 1.21	1.66 ± 1.21	2.16 ± 0.75

**Table 9:** Blood monocyte counts in Brucellosis affected animals treated with phage.

significant ( $p < 0.01$ ). However, the levels of leukocytes, monocytes and neutrophils did not differ significantly among themselves during the period of 90 days after treatment with the phage.

**Conclusion**

Our present study indicates that phage therapy in Brucellosis stimulates immune responses in cattle initially for about one and

a half month which decline slowly after 45 days of initiation of therapy.

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