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RESEARCH ARTICLE
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Therapeutic efficacy of Brucella phage against *Brucella abortus* in mice model

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Abstract

Aim: To investigate the preventive and therapeutic potential of brucella phage in experimentally infected mice with *Brucella abortus* (strain 544).

Materials and Methods: Three groups of mice each containing 6 individuals were infected with *Brucella abortus* (strain 544). A preventive group was infected with *Brucella abortus* (strain 544), 48 hrs before phage treatment. A therapeutic group was phage treated 48 hrs after *Brucella abortus* (strain 544) infection in mice. Spleen was aseptically collected from all groups of mice 15 days after challenge. Mean Spleen count of *Brucella* was enumerated by culturing on Brucella agar media (BAM) and converted in terms of protective activity and compared with control mice not receiving phage therapy.

Result: There was significant reduction in protective activity of preventive and therapeutic phage treated groups (2.884 and 3.077) respectively in comparison to control group mice (4.267). There was no significant effect on mice health following phage treatment.

Conclusion: phage treatment is a promising alternative for reducing *Brucella* colonization in mice.

Keywords: brucella phage, spleen count, phage therapy.

Introduction

Brucellosis is an important re-emerging zoonotic infectious disease [1, 2]. It is characterized by abortions, stillbirths and reproductive problems in ruminant [3]. Human beings get infected accidently by contact with vaginal discharges, fetal fluids [3] or by ingestion of unpasteurized milk [4]. Five out of nine species of *Brucella* can infect humans in which most pathogenic for human is *B. melitensis* and followed in descending order by *B. suis*, *B. abortus*, *B. canis* and recently isolated marine Brucellae *(B. ceti)* [5].

Bovine brucellosis is widespread in India and has been reported from almost all states [1,6]. An appropriate antibiotic therapy for animals and human beings is still disputed [7-9] and it is too expensive in most of the animal species. so use of lytic phage to treat brucellosis can be another alternative to this important disease

Phages are viruses that infect and multiply inside the bacteria and can have two types of life cycles, lytic and lysogenic [10]. The lytic phages are the most suitable for phage therapy as they quickly reproduce within and lyse the bacteria and grow exponentially in the number [11]. Bacteriophages are virus that use specific bacterial species as host, and reduce the number of viable bacterial cells without significant affecting commensal gut flora often caused by broad-spectrum antibiotics [12]. Phage replicates as long as the targeted host bacterium is present and thus they are naturally self-limiting [13]. Recently, phage therapy has regained general interest as resistance to antibiotics has become a serious problem [14,15] and is found to be effective to treat many bacterial infections [16-18].

The use of host-specific bacteriophages has been promoted as a cost-effective and adaptable approach to control zoonotic bacteria like *Brucella* and *Salmonella* [19] and have potential to be developed as effective agents to prevent and treat animal bacterial diseases [20, 21]. The objective of this study was to investigate the efficacy of phage treatment in reducing Brucella colonization and infection in mice.

Materials and Methods

Animals: Apparently healthy female Swiss albino mice (n=26) (4 to 6 weeks age) were obtained from the Laboratory Animal Resource section, Indian Veterinary Research Institute (IVRI), Izatnager. The animals were kept under conventional housing condition and provided feed and water ad labium.

Ethical approval: The animal ethics committee of Indian Veterinary Research Institute, deemed university approved the study.

Bacterial cultures: *B. abortus* 19, *B. abortus* 544, *B. melitensis* 16M, B. *melitensis* Rev 1, *B. suis* 1303 were obtained from the Brucella Referral Laboratory, Division of Veterinary Public Health, IVRI, Izatnagar. *Salmonella*, *Staphylococcus*, *E. coli* and *Pasturella*

used from Type Culture Lab, Division of Biological Standardization IVRI.

Phage lytic to *Brucella* spp: Phage lytic to *B. abortus* (strain 19) obtained from Department of Veterinary Microbiology, Veterinary College, Guru Angad Dev Veterinary and Animal Science University (GADVASU), Ludhiana, Punjab, India [22].

Preparation of phage stock: Working stock of isolated phage was prepared by seeding 500 ml flask containing 100 ml of NZCYM medium (Sodium chloride,Casein enzymic hydrolysate, Casein acid hydrolysate, Yeast extract, Magnesium sulphate, heptahydrate) (Himedia, Mumbai) with 0.5 ml suspension of *B. abortus* 19 harvested from 48 hrs grown culture on Brucella agar medium (Difco, USA) slant into sterile PBS (pH 6.8). Phage suspension added in this culture after 12 hrs (log phase) and incubation for 48 hrs at 37℃. Culture were centrifuged at 10000g for 10 min. Supernatant fluid was then sterilized by passage through a membrane filter (0.22µ, Millipore filter crop, U.S.A) and kept at 4℃. Titer (pfu/ml) of these phage stock determined by double overlay technique [23].

Test for toxicity of phage preparation: In separate experiments, the tested phage was administered i.p. (Intra peritoneal) to mice to determine the toxicity of phage in vivo. Three concentrations of the phage preparation (10^6 PFU/ml, 10^7 PFU/ml, and 10^8 PFU/ml) were tested on mice. Two mice were injected with 0.5 ml of each phage preparations along with 2 mice received 0.5 ml sterile PBS. A 7 day observation was made to see any toxic effect of phage preparation in terms of mortality and reduced weight gain.

Host range determination: The phage were tested for their ability to lyse different strains of *B. abortus* also with other species of the genus *Brucella*, *Salmonella*, *Staphylococcus*, *Pasturella* and *E.coli*. Bacteriophage lysis assay was based on the modified method of the traditional double-layer plaque technique [24]. The top agar layer consisted of NZCYM broth containing 0.75% agar. For each bacterial strain tested, 3 ml of top semisolid agar was melted for 10 min and allowed to cool to 47°C. After adding 100µl of overnight culture of bacterium to be tested on the melted agar, the melted mixture was vortexed and poured on a Brucella agar media plate. The top agar was allowed to solidify at room temperature and 30 µl phage suspensions (10^8 PFU/ml) were spotted onto the top layer. The plate was incubated at 37°C for 48 hrs and then examined for the presence of zone of lysis. (*B. abortus* 544 placed in 5% Co_2).

Examination of phage (Electron Microscopy): Phage-containing solutions (10^8 pfu/ml) were mixed with 1/10 volume of 2.5% buffered glutaraldehyde for 5 min. 25 µl of fixed phage were added to the surface of a piloform coated grid and left for 2 min. After removing excess fluid grid was washed for 2 seconds in distilled water. Negative staining of phage was performed by adding 1 drop of 2 % phasphotungstic acid (PTA) to the grid surface. After drying for 20 min in the air grids were observed with a JEOL transmission electron microscope [25].

Phage therapy trials: Three groups of six-week-old female Swiss albino mice each containing 6 individual were infected with 2 x 10^5 CFU *B. abortus* 544 in 0.1 ml PBS (pH 7.2) i.p. CFU determined as the method described by [26]. Preventive and therapeutic groups were treated with 10^6 cfu/ml of phage in 0.1 ml of PBS (7.2) i.p., 48 hrs before and 48 hrs after infection respectively [27]. Control groups of mice were inoculated with 0.1 ml PBS (7.2), i.p.

Splenic growth of challenge strain in Swiss Albino mice: All mouse were killed by cervical dislocation 15 days post-infection and spleens were removed aseptically, homogenized individually in 9 time to weight of spleen in buffered saline solution (BSS: NaCl 8.5 g; KH_2PO4 1.0 g; K_2HPO4 2.0 g; distilled water 1000 ml; pH 6.8) and three tenfold dilutions were done (1/10, 1/100 and 1/1000) in the BSS and 0.2 ml of each dilution was seeded in BAM (brucella agar media) plates (Difco, USA) (4 plate for each dilution, 2 kept aerobically and 2 kept in 5 % CO_2). Plates were incubated at 37°C for 4 to 5 days to determine the CFU/spleen; Colonies of Brucella should be enumerated on the dilutions corresponding to plates showing fewer than 300 CFU. When no colony was seen in the plates corresponding to the 1/10 dilution, the spleen was considered to be infected with five bacteria. These numbers of *Brucella* per spleen are first recorded as X and expressed as Y, after the following transformation: Y = log (X/log X) [28,29].

Statistical analysis: Data obtained in the study were analysed statistically on 'SPSS-16.0' software package as per standard methods [30]. Data were subjected to one way analysis of variance and level of significance among the treatments.

Results and Discussion

Phage was found lytic to culture of *B. abortus* 99, *B. abortus* 19, *B. abortus* 544, *B. melitensis* Rev1, and *B. suis* 1330, but not to *Salmonella abortus* equi E742 and E789, *Salmonella pullorum*, *Pasturella multocida* P_{52} and *Staphylococcus aureus* and *E. coli*. This finding is in accordance with findings of Chachra [21]. This observation showed that the phage was specific to *Brucella* genus and not to other gram +ve or gram –ve bacteria tested in the lab which give advantage that it might be not affect other bacteria flora in the body. Electron microscopy of the phage showed somewhat rounded head with a short tail, consistent with the previously report that most of brucella phage have short tails [31,32]. A titer of phage used in therapy was 10^6 PFU/ml. It comes into medium range of concentration that was suitable for phage therapy [33].

Table-1. Protective activity of phage in preventive group mice

Mice No.	Average total spleen colony count (X)			Protective activity		Average Protective activity (Y)
	1:10	1:100	1:1000	1:100	1:1000	
1	U	3500	2000	2.994	2.782	2.888
2	U	2000	1000	2.782	2.522	2.652
3	U	1200	3000	2.590	2.935	2.763
4	U	8000	7000	3.311	3.260	3.285
5	U	4400	4000	3.081	3.045	3.063
6	U	2000	1000	2.782	2.522	2.652

U= uncountable, Y= log (X/logX) where X= total CFU/spleen

Table-2. Protective activity of phage in therapeutic group mice.

Mice No.	Average total spleen colony count (X)			Protective activity		Average Protective activity (Y)
	1:10	1:100	1:1000	1:100	1:1000	
1	U	4000	1000	3.045	2.522	2.784
2	U	2500	2000	2.866	2.782	2.824
3	U	7500	9000	3.286	3.357	3.321
4	U	8000	7000	3.311	3.260	3.285
5	U	8200	7000	3.321	3.260	3.290
6	U	2500	4000	2.866	3.045	2.956

U= uncountable, Y= log (X/logX) where X= total CFU/spleen

Table-3. Protective activity of PBS in control group mice.

Mice No.	Average total spleen colony count (X)			Protective activity
	1:10	1:100	1:1000	
1	U	U	80000	4.212
2	U	U	120000	4.373
3	U	U	60000	4.098
4	U	U	75000	4.187
5	U	U	60000	4.098
6	U	U	230000	4.632

U= uncountable, Y= log (X/logX) where X= total CFU/spleen

Table-4. Camparision of mean protective activity of three group

Group	Mean Protective activity ± SE
Preventive	2.884 ± 0.103[a]
Therapeutic	3.077± 0.102[a]
Control	4.267± 0.084[b]

Means within each column bearing common superscript do not differ significantly (P< 0.05)
SE = standard error

Toxicity study of the phage preparation in mice showed that it was nontoxic to mice as there was no mortality and untoward reaction seen in the 7 day observation period after administration.

Spleen count of *B. abortus* 554 did 15 days after administration of phage [28, 29]. It was found that the mean protective activity of phage was 2.88, 3.08 and 4.27 in protective, therapeutic and control group's mice respectively (Table-1, 2, 3 and 4). Protective activity was significantly (P<0.05) decrease in the preventive and therapeutic group as compared to control group (Table-4). It means that there is significantly reduced in spleen count of both phage treated group as compare to the non treated control group. This observation was supported by the work of Parnas; Pamas and Burdzy [34-36] who established that brucella phage possesses certain preventive properties against experimental challenge. The reduction in colony count of *Brucella* was found to be higher in the 1st group, in accordance with the finding of Drozevkina; Pophadze [37, 38] who demonstrated that the introduction of the phage into the organism at the same time or immediately after infection lead to the localization of the causative agent and increase the sterilization of the organism. Reduction in the spleen count of *Brucella* with of a single dose of phage after 15 DPI suggest that the phage could be stable and active in vivo for a longer duration This finding is also supported by the work of Pophadze [39] who showed that brucella phage is capable of persisting for a long time both in a healthy guinea pig (up to 30 days) and in a guinea pig infected with brucellosis (over 45 days). Recently many trails of phage therapy in mice, poultry and cattle showed that phages are effective promising alternatives to bacterial infection [40, 41].

Conclusion

It is clear from this study that application of phage is an effective mean to reduce the colonization of *Brucella* in the spleen of mice. Since the use of antibiotic in brucellosis in animals was uneconomical and not recommended, the use of the phage to treat the animal especially pregnant animal may help in reducing the chance of threatened abortion and potential transmission to other susceptible animal. This is the preliminary work in this field, and further study is required to find a more appropriate route, dosage and time interval to get maximum result and more experiments are needed to make this therapy applicable to all conditions of brucellosis.

Authors' contributions

MR designed the study and all the other author performed research experiment. AP and HV carried out the statistical analysis. MR drafted and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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