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Therapeutic Efficacy of Lytic Bacteriophage PSAE-1 against *Salmonella* Abortusequi in Guinea Pig Model

Abhishek[1], R.K. Agarwal[1], B. Kumar[2], A.K. Mishra[3], Anjay[4], A. Priyadarshini[1], S. Kumar[1], C. Prakash[1], J.K. Chaudhary[5] and M. Rawat[6]

[1]Division of Veterinary Bacteriology and Mycology, Indian Veterinary Research Institute Izatnagar, Bareilly (U.P.) – 243122, India.
[2]Division of Biological Products, Indian Veterinary Research Institute Izatnagar, Bareilly (U.P.) – 243122, India.
[3]Goat Health Division, Central Institute for Research on Goats (CIRG), Makhdoom, Farah-281122.
[4]Division of Veterinary Public Health, Indian Veterinary Research Institute Izatnagar, Bareilly (U.P.) – 243122, India.
[5]Division of Livestock economics, statistics and Information Technology Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.) – 243122, India.
[6]Division of Biological Standardization, Indian Veterinary Research Institute Izatnagar, Bareilly (U.P.) – 243122, India.

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Global emergence of multi drug resistance (MDR) against the bacterial pathogens has motivated scientists to exploit alternative strategies to combat the problem. Under these demanding circumstances, therapeutic uses of bacteriophages may turn out to be a valuable approach. The present study was planned to evaluate therapeutic efficacy of *Salmonella* Abortusequi phage (PSAE-1) in experimentally infected female guinea pig. Phage PSAE-1 was characterized in terms of lytic host range and multiplicity of infection (MOI). Broad host range phage having MOI of 1:10^4 was used for therapeutic trial. Safety of phage was tested in experimental animal for any toxicity and it was found safe through various routes. Therapeutic efficacy of PSAE-1 was assesed in female guinea pig at different time intervals after infection. Average total spleen counts of *Salmonella* Abortusequi strain-156 (SAE-156) were enumerated after the phage treatments. There was significant increase in therapeutic response of phage treated groups in comparison to untreated control group. There was no significant effect on animal health following phage treatment. Present study suggests that phage may be used in future as a therapeutic preparation to reduce the colonization/infection of *Salmonella* Abortusequi and could be a novel approach in treatment of infections associated with *Salmonella* spp.

Keywords: *Salmonella* Abortusequi, Bacteriophage, Guinea pig, Therapeutic.

Salmonella enterica subspecies *enterica* serovar Abortusequi, an host-adapted *Salmonella* serovar recognized as a cause of late term abortion in mares, still birth, birth of weak and debilitated new born and a range of other clinical conditions leading to huge economic losses to the equine breeders and stud owners[1,2]. *Salmonella* Abortusequi not only associated with genital tract infections but also leading to major breeding inconsistencies and turning up fertile mare to barren[3]. A number of antibiotics are available in the market against the infections caused by *S.* Abortusequi and other *Salmonella* sp. But, in recent time due to indiscriminate use of antibiotics, multi drug resistant *S.* Abortusequi isolates of Indian origin have been discovered and these isolates have also shown resistance against the

* To whom all correspondence should be addressed.
Tel.: +91-9456600623;
E-mail: abhivbm@gmail.com

antibiotics that were not even used earlier[4]. Thus, the global emergence of MDR against the bacterial pathogens and their mild response to various antibiotics has motivated scientists to exploit alternative anti-infection strategies to combat the problem. Under these challenging circumstances, therapeutic uses of bacteriophages may turn out to be a valuable approach. Bacteriophages are the viruses infecting bacteria and have great potential to be used as therapeutic agents against the bacterial diseases. Use of phages against MDR bacteria, opens up a new possibility of treating bacterial diseases[5]. Phage therapy has been proved successful and has emerged as a promising agent in the treatment of bacterial infections including *Salmonella*[6,7]. In view of the problem of drug resistance and success reports of phage therapy, the present study was planned to evaluate safety and therapeutic efficacy of the bacteriophage against *S*. Abortusequi in guinea pig model.

MATERIALS AND METHODS

Bacterial strains

Salmonella Abortusequi strains E-156 (SAE-156), E-155, E-157, E-742 and E-789 were procured from Division of Biological standardization, IVRI, Izatnagar. Master seed lots of all strains were prepared after their revival, purification and confirmation by biochemical and serological tests. Viable counts of SAE-156 were carried out by standard serial dilution method in terms of Colony Forming Unit (CFU/ml). The organism was passaged 3 times in guinea pig to increase it virulence and re-isolation of the organism was done from heart blood, *Salmonella* Typhimurium, *Salmonella* Entertidis, *Salmonella* Pullorum E-79, *Salmonella* Gallinarum, *Escherichia. coli* used for host range determination of PSAE-1 were procured from National Salmonella Centre, Bacteriology and Mycology Division, IVRI, Izatnagar and *Pasturellla multocida* from B&M Division IVRI, Izatnagar whereas *Staphylococcus aureus*, and *Streptococcus* sp from Type Culture Lab of Division of Biological Standardization, IVRI, Izatnagar.

Bacteriophage

Previously isolated *Salmonella* Abortusequi phage (PSAE-1)[3] used for the present study were procured from Division of Biological Standardization.

Animals

Apparently healthy female adult guinea pigs (Dunkin-Hartley) were procured from LAR section, IVRI, Izatnagar. The animals were kept under conventional housing condition and provided feed and water ad libitum.

Revival, purification and preparation of phage stock

The stock suspension of the phage PSAE-1 was filtered by passing through 0.22 µm PVDF filter (Millex-HV) and tested for presence of lytic activity against the selected SAE-156 by spot test as per the method by Rahaman *et al.*[8] and O'flaherty *et al.*[9] with some modifications. Briefly, 100 µl of the 6-8 hrs pure growth of SAE-156 isolate in NZCYM broth (NewZeland Casamino Yeast Medium: Sodium chloride, Casein acid hydrolysate, Yeast extract, Magnesium sulphate-Difco USA) was spread over the surface of separate NZCYM agar (2% agar) plate. After 5 minutes, 4 µl of filtered phage suspension was aseptically placed over a surface of the agar and incubated at 37ºC for 16-18 hrs. The plates were observed for zones of clarification (lytic zone). Phage PSAE-1 was further confirmed and tested for ability to produce plaque by soft agar over lay method[10]. Plaques were further purified by liquid culture method[11]. The purified phage was harvested by using about 2 ml of Salt Magnesium buffer (SM buffer: 50 mM Tris-Cl [pH: 7.5], 0.1 M NaCl, 8 mM MgSO. $7H_2O$, 0.01% Gelatin). The harvested suspension was clarified, filtered and used for future experiments.

Host range of PSAE-1 and enumeration of plaque forming unit (PFU)

Host-range of purified phage PSAE-1 was determined by spot test as described earlier[9] against different homologus strains of *S*. Abortusequi (E-156 (SAE-156), E-155, E-157, E-742 and E-789) and heterologus strains of *Salmonella* viz; *S*. Typhimurium, *S*. Entertidis, *S*. Pullorum E-79, and *S*. Gallinarum. PSAE-1 was also tested for across genus lytic activity against *E. coli*, *S. aureus*, and *Streptococcus* sp. The phage count of the PSAE-1 was determined by combination of standard serial fold dilutions method and agar overlay technique in terms of plaque forming unit (PFU).

Determination of multiplicity of infection (MOI) of phage

The optimum phage to bacteria ratio required to achieve 100% lysis of the indicator strain within shortest period of time termed as MOI of the phage. MOI was conducted as per the method of Mishra *et al.*[12] with slight modifications. The lowest phage-bacteria ratio which showed complete lysis of the organism or highest lytic activity in shortest period of time was considered as MOI of PSAE-1.

Generation of therapeutic phage preparation

Phage preparation intended for therapeutic use was produced employing standardized optimum conditions. Briefly, 0.1 ml of suspension of 14-16 h old SAE-156 seeded in 100 ml NZCYM broth. After 24 h, a volume of phage PSAE-1 was directly added in culture broth, so that phage: bacteria ratio became equivalent to MOI and incubated at 37°C with intermittent shaking until the turbidity of suspension was reduced to about 90%. Preparation was centrifuged at 8000-10000g for 15 min and filtered through 0.22 ìm PVDF filter. This filtrate was used as therapeutic preparation in animal experimentation.

Safety of preparation in guinea pig

Safety tests of PSAE-1 preparation intended for *in vivo* trials were conducted in guinea pigs as per method of Abhishek *et al.*[13] with modifications. Six adult healthy guinea pigs two in each injected with a 1 ml of Phage containing 10^8 PFU/ml through intra muscular (I/M), subcutaneous (S/C), and intra peritoneal (I/P) route respectively. Any untoward reaction or mortality was noted up to 20 days. Endotoxin content of preparation was determined by LAL assay using commercially available Kit (Lonza) as per the instructions of the manufacturer.

Therapeutic efficacy (TE) trial of phage

The therapeutic trail of the phage PSAE-1 was determined as per method of Prajapati *et al.*[14] with modifications. Four group (P_{UT}-Phage untreated, P_{T6}-Phage treatment at 6 hrs, P_{T12}- Phage treatment at 12 hrs and P_{T24}- Phage treatment at 24 hrs) of healthy adult female guinea pig each containing six animals were infected with 10^7 of SAE-156 in 0.1 ml of Phosphate buffer saline (PBS: NaCl. 8.5 g; KH PO_4 1.0 g; K HPO_4 2.0 g; distilled water: 1000 ml; pH 7.2). by I/P route. Each animal of P_{T6}, P_{T12} and P_{T24} group received 10^5 PFU/of phage in 0.1 ml of PBS at different time intervals. P_{UT} group inoculated with 0.1 ml of PBS I/P (Table1). All animals were sacrificed humanly under CO_2 and chloroform method 6 days post-infection and spleens were aseptically collected, weighed and homogenized individually in 9 times to weight of spleen in sterilized PBS. Four serial tenfold dilutions (1/10, 1/100, 1/1000, and 1/10000) of the homogenate were made. 0.1 ml of each dilution was spread plated on Hektoen Enteric agar (HE agar medium: Difco, USA) plates, incubated at 37°C for 12 hrs and average total spleen counts were determined. Numbers of *Salmonella* per spleen was first recorded as X and expressed as Y after the following transformation Y= log (X/log X) [14]. The data of present study were analyzed using 'SPSS 20'software package using one way analysis of variance and level of significance among the treatments.

RESULTS AND DISCUSSION

All *S.* Abortusequi strains used in the present study, on revival exhibited typical morphological, biochemical and serological characteristics (4, 12: e, n, x antisera) of *S.* Abortusequi (Fig1a and 1b). The average viable count of 12-14 h incubated NZCYM broth cultures of selected indicator strains SAE-156 was determined as ~10^8 CFU/ml.

Lytic range of phage is important criteria in the selection of phage intended to be used for therapeutic application. Phage PSAE-1 was found lytic to all *S.* Abortusequi strains tested but not to other heterologus strains of *Salmonella* viz *S.* Typhimurium, *S.* Entertidis, *S.* Pullorum E-79, *S.* Gallinarum and other bacterial pathogens *E. coli, P. multocida, S. aureus*, and *Streptococcus sp.* The first mandatory step for developing a universal phage-based therapeutic system is the availability and ability of phages that can lyse the target pathogen/pathogens. Phage PSAE-1 was reoprted to be first lytic phage against *Salmonella* Abortusequi in India[3] was revived, purified and produced in bulk. Phage PSAE-1 showed clear lytic zone against SAE-156 and other strains by spot test (Fig 2) and produced clear plaques (Fig 3). The phage count/titre (PFU/ml) of the phage PSAE-1 was found to be 10^8 PFU/ml.

The effectiveness of the phages in reducing bacterial colonization/infections is depending on phage concentration and it may vary at different incubation temperatures and duration of incubation[15]. Therefore, to standardize the optimum phage to bacteria ratio an experiment on MOI was conducted. The MOI of phage required to achieve 100% lysis of the indicator strain SAE-156 within 180-200 minutes was $1:10^4$. In a similar study by Ramchandra *et al.*[16] who reported optimum lytic efficacy of *Pasturella* phage against *P. multocida* was 1:100. The findings of Mishra *et al.*[12] supports our work who reported that minimum of 0.01 MOI is required for optimum lysis of *Staphylococcus aureus* associated with goat mastitis. Hungaro *et al.*[7] conducted MOI trails on different temperatures reported significant difference in the reduction of growth of *Salmonella* Entertidis depending upon MOI. The study on MOI of phages indicates that it may vary depending upon the phage and the target bacteria.

About 75 ml of the therapeutic preparation was generated employing standardized optimum conditions and re-tested for presence of lytic effect by spot test. The endotoxin content of preparation was found less than 2 EU/ml.

In clinical settings, phages appear to be safe as there have been no reports of complications associated with their use but it becomes imperative to ascertain the safety of phage before subjecting

Table 1. Schedule for challenge and treatment of phage.

Group	No of animals	Challenge dose (0.1 ml)	Route of administration	Time of phage treatment after challenge (0.1ml) 10^5 PFU/ml, I/P
Untreated control P_{UT}	6	10^7 CFU/ml	I/P	No phage treatment
P_{T6}	6	10^7 CFU/ml	I/P	After 6 hrs
P_{T12}	6	10^7 CFU/ml	I/P	After 12 hrs
P_{T24}	6	10^7 CFU/ml	I/P	After 24 hrs

Table 2. Total spleen counts of *Salmonella* Abortusequi of individual guinea pig

Group Animal no	Total spleen counts(X) of *Salmonella* Abortusequi at 1:1000 dilution					
	1	2	3	4	5	6
Untreated control (P_{UT})	240000	285000	NC	290000	288000	227000
P_{T6}	60000	10000	18000	34000	30000	29000
P_{T12}	7000	4000	7000	13000	15000	9000
P_{T24}	73000	67000	97000	23000	19000	37000

Table 3. Transformation value (Y) = log(X/logX) for different treatment groups

Group	Transformation value (Y)=log(X/log X) of each guinea pig in groups						Mean	Therapeutic response=(Mean Y control - Y treated)
Animal no	1	2	3	4	5	6	Y±SE	
Untreated control (P_{UT})	4.649412	4.718062	NC	4.725015	4.722248	4.627183	4.68 ± 0.020^{c}	Nil
P_{T6}	4.098891	3.39794	3.626345	3.875239	3.826122	3.81283	3.77 ± 0.097^{ab}	0.91
P_{T12}	3.260191	3.045509	3.260191	3.357179	3.555321	3.499685	3.32 ± 0.075^{a}	1.36
P_{T24}	3.908456	4.142481	4.288952	3.722069	3.647436	4.17639	3.98 ± 0.106^{b}	0.70

Different superscript a, b differ significant (P>0.05)
Mean value bearing Different superscripts (a, b, c,) differ significantly (P>0.05)

them to *in vivo* trials. Safety test of the Phage PSAE-1 was conducted in guinea pigs and it was found safe through various routes. The present study showed the concordance with the previous studies conducted on mice and human to test the safety of *E. coli* T-4 phages[17,18]. Likewise, Chilamban *et al.*[11] reported that *Staphylococcus aureus* phage do not showed any untoward reaction in mice through intra-mammary route. The findings of Carlton *et al.*[19] are also in accordance with our observations who tested safety of Listeria-phage P100, in rats with high doses and no measurable effects. Abhishek *et al.*[13] reported that crude & purified phage preparations, as well as lytic enzyme of phage SA1 against *Staphylococcus aureus* (IVRI-82) did not showed any adverse effects on mice through subcutaneous route. None of the reports mentioned significant phage related undesirable side effects. But, it would be prudent to ensure further the safety of therapeutic phages before using them as therapeutic agents.

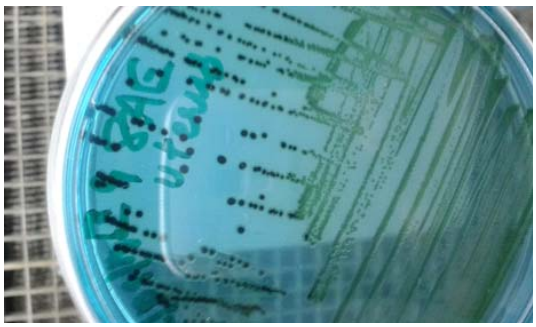
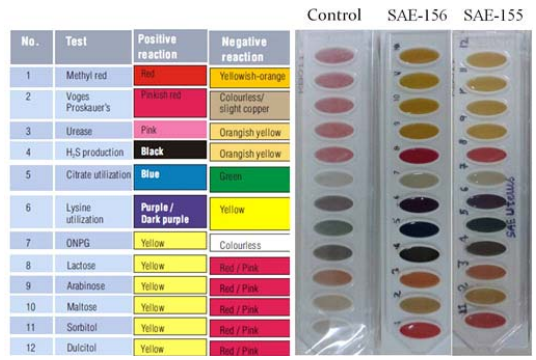


Fig. 1. a) Black centered colony with greenish periphery of *Salmonella* on HE agar; b) Biochemical confirmation of *Salmonella*



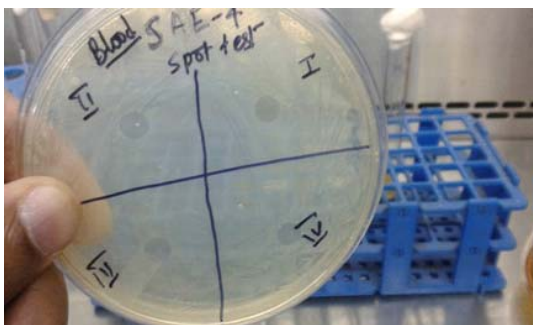


Fig. 2. Spot test: lytic zone of phage PSAE-1 on bacterial lawn

In recent time bacteriophage therapies have been reemerging as potential candidate as an alternate to antibiotics[20,21]. Phages have been used widely for treatment of several bacterial infections including *Salmonella* infections[22]. Purified phage PSAE-1 was administered at different time interval viz. 6 hour, 12 hour and 24 hrs after challenge with a dose of 10^5 PFU/ml through I/P route. Therapeutic index of phage treated group are significantly higher (P_{T6}: 0.91, P_{T12}: 1.36, P_{T24} : 0.70) in comparison to untreated control (Table 3). Phage treatments at different time duration are significantly reducing the average total spleen counts of infected organism but the reduction is maximum when the therapeutic inoculation of phage was given at mid phase of infection i.e with in 12 hrs (Table 3). The average total spleenic counts of group P_{T6} and P_{T24} were significantly reduced as compare to

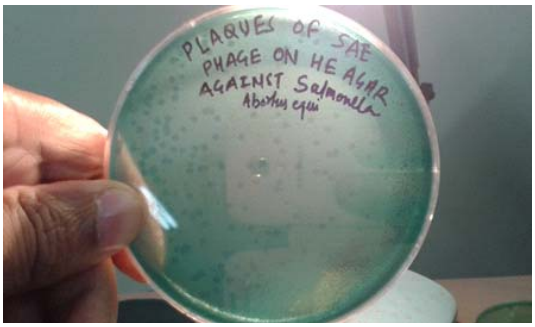


Fig.3. a) Plaque by PSAE-1 over nutrient agar plate; b) Plaque by PSAE-1 over HE agar plate

untreated control (P_{UT}) though this reduction was little bit more significant in P_{T6} group (Table 2). All untreated-challenged controls showed presence of its challenge organism in spleen in a range $>10^6$. Observations of our study is supported by the work of Smith and Huggins[23], who reported that anti-K1 bacteriophages were able to lyse K1+ *E. coli* when administered by intramuscular injection at the same time or eight hours after, infection with *E. coli*. Biswas *et al.* [24] reported that vancomycin-resistant *Enterococcus faecium*-infected mice were successfully treated with phage therapy just 45 minutes after infection and five hours after infection but only 50% recovery was seen up to 18 or 24 hours infection. Capparelli *et al.* [25] found that 100% of mice were died in control group challenged with a lethal dose of CFU (10^7) of *S. enterica* serovar Paratyphi B whereas all mice in phage Ø 1 treated group were survived. The observations of therapeutic trial of Phage Lys SA4 in mice by Mishra *et al.*[26] partially supports our findings who reported that mean viable count of *Staphylococcus aureus* in the spleen of phage treated mice were considerably low when phage treatment was given after 24 hrs of challenge. Hong *et al.*[21] reported that mortality and re-isolation of *Salmonella* Gallinarum decreased significantly in challenged chickens treated with the bacteriophages. The findings of Prajapati *et al.*[14] on Brucella phage in reducing spleen count of phage treated group after 48 hrs of challenge with *B. abortus*544 partially supports our work. Recently, large no of studies on phages has been conducted against various bacterial agents have proved the therapeutic potentials of lytic phages[22]. Our work supports past studies and indicates that further research with *Salmonella* Phages may widen the scope of useful applications of this biological agent in field conditions as therapeutics.

CONCLUSION

The Present study indicates that phage can be used as an effective mean to reduce the colonization of *S.* Abortusequi in the guinea pigs. Phage preparation was found safe in experimental animals through various routes of inoculation at the titre of 10^8 p.f.u/ml. Therapeutic trails suggest that phage preparation can be used for first phase trials in target animals. Preliminary studies are encouraging and further study is required to establish more safety, specificity, appropriate dosage and long term effectiveness of phages in target animals.

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Ethical approval

The animal ethics committee of (IAEC) Indian Veterinary Research Institute, deemed university approved the study. All animals were kept under standard management conditions and sacrificed at the end of experimentation for ethical reasons by standard method recommended by IAEC/CPCSEA.

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