ORIGINAL ARTICLE

BACTERIOPHAGE VERSUS DRUG RESISTANT BACTERIA – AN APPROACH TO ALTERNATIVE THERAPY USING AN EXCISION WOUND ANIMAL MODEL.

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ABSTRACT

Treatment of infections due to methicillin resistant *Staphylococcus aureus* is a matter of great concern since the past few decades. Alternatives using phages can be a potential therapeutic option due to higher degree of host specific, pharmacokinetic and pharmaco-dynamic properties compared to antibiotics. The present study was conducted to evaluate the efficacy of phages against MRSA 43300 in diabetic animal model. Phages were isolated from sewage and anti-MRSA activity was confirmed *in vitro*. Excision wounds of diabetic rats were infected with various doses of MRSA 43300 and challenged with various doses of phage lysate to determine the minimum lethal dose (MLD) of bacteria and minimum protective dose (MPD) of phage respectively. MLD of $4x10^7$ CFU/mL of MRSA 43300 was able to establish severe illness in rats after 24h of infection and all the animals recovered and survived at an MPD of $3x10^9$ PFU/mL of phage. Rising numbers of multidrug resistant organisms in the recent times has enforced into looking for alternative therapeutic options and the present study indicates that phage therapy is one of the prospective options which need further research on a larger scale.

Keywords: Diabetes, Excision wound, MRSA, Phage therapy

INTRODUCTION

Diabetes mellitus has emerged as a major problem in healthcare in the past few decades. Treatment of the diabetes associated complications, especially foot ulcers is challenging due to the underlying metabolic disorder. Diabetic foot ulcer (DFU) is one of the major causes of amputations of the affected part, increasing the morbidity and mortality among diabetic patients. Infection of DFU is usually poly-microbial, organisms are often multidrug resistant. Methicillin resistant Staphylococcus aureus (MRSA) is one the most commonly associated pathogens. Treatment of such infections is usually challenging, due to limited available choices, which calls for looking into alternative therapeutic options.[1-3]

Phage therapy is a promising alternative to antibiotic therapy in recent times as observed by few researchers.[4,5] Phage therapy can be a potential alternative due to the pharmacokinetic and pharmaco-dynamic properties.[6] Route of administration, number of doses and duration of treatment are also favorable in case of phages compared to antibiotics.[7] The present study was aimed at studying the efficacy of phages isolated from sewage against MRSA infected diabetic excision wound animal model.

MATERIALS & METHODS

Study setting and ethical clearance: The present study was conducted at the Institutional Animal Facility, Department of Pharmacology and Microbiology, S.S. Institute of Medical Sciences & Research Centre, Davangere, India. The study had the approval of Institutional Animal Ethics Committee of S. S. Institute of Medical Sciences & Research Centre and Institutional Biosafety Committee of S. S. Institute of Medical Sciences & Research Centre, Davangere, India.

Bacterial Isolate: MRSA ATCC 43300 was used as the bacterial control.

Experimental animals: Twelve weeks old healthy albino rats (180-200 gm) of either sex, bred locally were used for the study. The rats were maintained as per National Institute of Health (NIH) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPC

Isolation of bacteriophage: Phages were isolated from domestic sewage and hospital effluent treatment sites, Davangere, India. Isolation and purification of phages was done as described by Smith et al and Sambrook et al.[8,9] Spot inoculation on MRSA lawn culture and Soft agar overlay was used for analyzing the in vitro bactericidal activity of bacteriophage against MRSA 43300.[10]

Induction of diabetic wound in rats: Diabetes was induced chemically by administering streptozotocin (STZ) (65 mg/kg in 0.1M sodium citrate) intraperitoneally, following a fasting period of 12 h.[11] Rats were inflicted with excision wounds on the dorsal interscapular region, 1cm away from the ears according to the method described by Morton and Malone.[12] A circular full thickness excision wound (225 mm2 wide, 2mm deep) was created. Ketamine hydrochloride (25 mg/kg body weight) was used as the anaesthetic agent.

Determination of minimum lethal dose (MLD) of MRSA 43300: The MLD of MRSA 43300 was determined based on a previous study[4] The study suggested the MLD of MRSA as $4x10^7$ CFU/mL. Three groups of five animals each was infected with three doses of MRSA 43300 (i.e., Group I- $4x10^6$ CFU/mL, Group II- $4x10^7$ CFU/mL, Group III - $4x10^8$ CFU/mL). The animals were observed over 48h for signs of infection and were graded for the severity of illness.[13]

Determination of minimum protective dose (MPD) of phage: The purified phage lysate was serially diluted and inoculated onto the MRSA infected excision wound. Based on a previous study (the MPD of phage vs MRSA was identified as $3x10^9$ PFU/mL), 3 groups of 4 diabetic excision wound animal models infected with MLD of MRSA 43300 were challenge with 3 doses of phage (i.e., $3x10^8$ PFU/mL, $3x10^9$ PFU/mL, 3x1010 PFU/mL). [4] The animals were observed for progression of or recovery from illness for a period of ten days. The phage titre at which 100% of the animals survived was considered as the minimum protective dose of phage vs MRSA 43300.

RESULTS

Diabetes was induced in all the animals following a single dose of streptozotocin after 48h. (Fasting blood glucose was > 300mg/dL). Excision wound was created and inoculated with various doses of MRSA 43300. The results are shown in Fig 1.

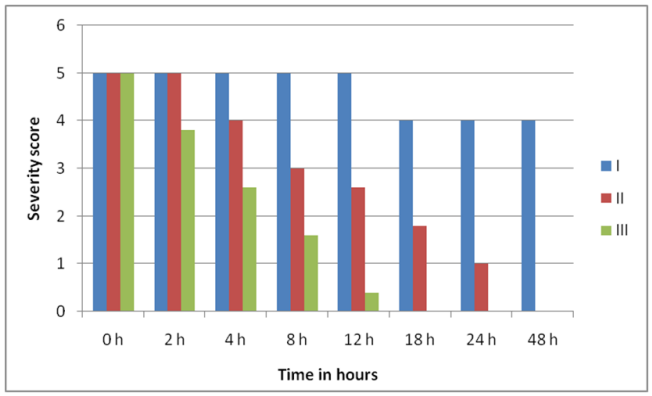

Fig 1: Clinical condition of animals at various MLD of MRSA 43300: [5 - Unremarkable condition; 4 - Slight illness (Ruffled fur and lethargy); 3 - Moderate illness (Hunched back, ruffled fur, severe lethargy); 2 - Severe illness (Scores 3 +4+ pus at the site of inoculation); 1 - Moribund state; 0 – Death]

Severe illness along with pus at the wound site was seen at a bacterial concentration of $4x10^7$ CFU/mL. All the animals died after 48h. Slight illness was seen at MRSA $4x10^6$ CFU/mL. All the animals died within 24h at $4x10^8$ CFU/mL. MLD of MRSA was identified as $4x10^7$ CFU/mL.

Three groups of wounded diabetic animals were challenged with MLD of MRSA i.e., $4x10^7$ CFU/mL and challenged 3 doses of phage. The results are shown in Table 1. All animals died by day 8 at a phage titre of $3x10^8$ PFU/mL. However, all the infected animals survived when challenged with phage $3x10^9$ PFU/mL and $3x10^{10}$ PFU/mL. Since all the animals recovered and survived at a minimum bacteriophage concentration of $3x10^9$ PFU/mL, it was considered as the MPD of phage against MLD of MRSA 43300.

DISCUSSION

MRSA infected diabetic wound infections impose a great health care burden due to the virulence factors, genetic properties and drug resistance capacity of the infecting organism. Phage therapy can be one of the main stay for treatment of such chronic infection. In the present study, diabetic animal models infected with MRSA 43300 were used. MRSA 43300 is a ATCC strain and hence the results of MLD can be reproduced at different settings. We observed that, a single dose of phage as low as $3x10^9$ PFU/mL was sufficient to save all the animals infected with a dose of MRSA as high as $4x10^7$ CFU/mL.

Table 1: Effectiveness of different doses of phage on MLD of MRSA 43000 in diabetic excision wound animal model.

Groups	No. of animals survived									
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
I $3x10^8$ PFU/mL	4	4	3	3	2	2	1	0	0	0
II $3x10^9$ PFU/mL	4	4	4	4	4	4	4	4	4	4
III $3x10^{10}$ PFU/mL	4	4	4	4	4	4	4	4	4	4

A previous study in our setting by Vinodkumar CS et al has reported similar results with regard to MLD of clinical MRSA isolates and MPD of phage isolated from sewage.[4] Our study reconfirms the findings of the previous study. This may indicate that there are no major changes in the infection rate of MRSA and phage dynamics among phages isolated in this part of the country.

For phage therapy, pharmacokinetics is analogous to the change in phage densities in different tissues of the host; and pharmaco-dynamics is analogous to the population dynamics of the phage–bacterial interaction. There is a profound difference in the population kinetics of phage relative to the pharmacokinetics of antibiotics because phages can replicate. If the target population of bacteria is sufficiently dense, treatment with just a few phage particles can result in profound increases in phage densities in tissues.[7,14]

Various studies have reinforced the use of phages for treatment of infections.[4,5,13] Our study reiterates that phages need to explored as one of the important sources of alternative therapy, especially in cases of infections with limited treatment options.

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