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RESEARCH ARTICLE

EFFECT OF TEMPERATURE AND PH ON THE STABILITY OF BACTERIOPHAGE AGAINST *STAPHYLOCOCCUS AUREUS* CAUSING MASTITIS

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ABSTRACT

An important disease that is economically important among the dairy cattle is mastitis. Its control is somehow complicated by high standard resistance to antibiotics. Hence, there is alternative way to control bacterial infection and contamination by phage therapy. In the present research we checked the effect of temperature and pH on the stability of bacteriophage against *staphylococcus aureus* causing mastitiswhich is isolated from the sewage water of dairy farm. The bacteriophage showed lytic activity against these bacteria. The lytic activity of bacteriophage at varying temperature and pH established their stability range while their highest lytic activity was observed at 37 C and pH 7.0. The storage condition of -20 C was proved to be best for the phage. The phage efficiently reduced bacterial growth in the bacterial reduction assay.So it signifies the underlying potential of bacteriophage therapy.

INTRODUCTION

Mastitis which is the Inflammation of parenchyma of mammary glands which is the most common reason of death in adult dairy cows.(Sudhan and Sharma 2010).It is considered as the most frequent production disease in the developing countries(Rajala-Schultz, Gröhn *et al*. 1999; Seegers, Fourichon *et al*. 2003)this disease cost the loss of 35$ billion US dollars per year. Especially staphylococcal mastitis can be manifested clinical and subclinical infection that can retain throughout lactation period.(Green and Bradley 2004; Han, Kim *et al*. 2013).Antibiotic therapy commonly does not treat such infection in a satisfactory way to remove existing disease or to cope with the establishment of chronic infection. Moreover the pathogenic potential of staphylococcus is very much aided by its ability to have resistance to antibiotics. In 1920 bacteriophages were researched as antibacterial agents to remove bacteria including staphylococcus in human infection and the result of wide range therapy research have been completely reviewed.(Yang, Liang *et al*. 2010)(Li and Zhang 2014)(Sulakvelidze, Alavidze *et al*. 2001). It is clear that recently the exploitation of phage as antibacterial agents has been experimented, a proof of interest and research with many pathogenic bacteria getting targeted.(Levy and Marshall 2004; Li and Zhang 2014)(Rose 1996; Smith, Pearson *et al*. 1999; Thacker 2003; Dixon 2004; Levin and Bull 2004; Thiel 2004).Lytic phages are similar to antibiotics in that they have remarkable antibacterial activity (Harper and Enright 2011).

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MATERIALS

The composition of media used was in gram/liter unless otherwise specified according to the requirement. Sterilization was done by autoclaving at 121^0C and 151b./ $inch^2$ for 15 minutes. Solution was filtered by using syringe filter of 0.2 µm. 0.1 M HCl and 0.1 M NaOH were used to adjust the pH of media. All glass-ware was washed and cleaned with detergent and then sterilized in autoclave, dried variably at 60-100^0C.

Glass ware used

Incubater	At 37^0C
Test tubes	20 ml
Ependorf	1.5 ml
Micropipette	1000 µl, 500 µl, 100 µl
Micro tips	1000 µl, 500 µl, 100 µl
Centrifuge	11000 G
Filter paper assembly	0.45µl
Flask	25ml

Chemicals and media

2 X L-Broth

Table 2.2. To prepare 2 XL-broth add 40 gram in 1000 ml of distilled water and autoclaved it

Serial No.		Components	Gms/L
1	1.	Tryptone	20
2	1.	Yeast Extract	10
3	2.	NaCl	10

Fresh culture of *staphylococcus aureus*

Strain.	*staphylococcus aureus*

L-Agar

35 gram L-agar is dissolved in 1000 ml of distilled water and autoclaved it.

Table L – Agar 1000 ml

S. No.	Components	gms/L
1	Tryptone	10.0
2	Yeast Extract	5.0
3	NaCl	5.0
4	Agar	15.0

METHODS

Sampling

Sampling was done from sewage and the liquid manure from cowsheds and main drain of township.

Host Bacterial Strains

This study included different bacterial strains that are *Staphylococcus aureus*. As the host strains for the characterization of bacteriophages against them was kindly provided by Dr. Noman from department of microbiology and molecular genetics university of Punjab.

Phage Titer Determination

Phage titer was determined as plaque-forming units (PFU/ml). Single isolated plaque was enriched according to the previous method and prep was again serially diluted and 10 ul from each dilution was mixed with 140 ul bacterial culture and 850 ul L-broth and over layering was done after mixing with the soft agar on agar plates. Incubation at 37c was given overnight and number of plaques was counted and PFU/ml was calculated according to the following formula (Capra, Quiberoni *et al*. 2006)

PFU/ml = NO. of plaques × dilution factor

Plaque Morphology

After over-layering morphological characterization of plaques was done by observing their size and shape.(Capra, Quiberoni *et al*., 2006).

Effect of pH on Stability of Bacteriophages

Development and survival of microorganisms are fundamentally affected by the pH of nature's domain and all microorganisms contrast in their necessity. Bacteriophages can develop inside a particular pH range which may be wide or constrained. This test was performed to check the impact of pH on the practicality of the disengaged bacteriophages. The arrangement of pH values over which any substance develops is communicated by 3 essential focuses: the base ph (the life form does not develop underneath it), the maximum pH (the organic entity does not make due over that esteem), and the ideal pH (the living being develops finest). To perform this test, pH of the L-broth was balanced at diverse pH levels i.e., 3-12. L-broth was spilled in distinctive Ependorfand a known centralization of purified bacteriophages was incubated at these pH levels for one hour at 37°c. The incubated phages were then blended with exponential development culture of the host microorganisms and incubated at 37°c for 15 minutes. The incubated mixture was then plated by double layer agar method technique. Test (for every pH and phage) was repeated twice**.**

Effect of Temperature on Stability of Bacteriophages

Microbial development is straightforwardly reliant on temperature and any change in this parameter represses development by influencing catalyst action. A certain microorganism will show an arrangement of temperature values over which it develops, which is communicated by 3 fundamental focuses correspondingly as in ph. The impact of temperature on bacteriophages was examined at distinctive temperatures i.e., 30, 37,50,70,80 and 100°C. L-broth was spilled in distinctive eppendorf and a known concentration of purified bacteriophages was incubated as officially said above at diverse temperatures for one hour. The incubated phages were then blended with exponential developmental culture of the host microorganisms and incubated at 37°C for 15 minutes. The amount of plaques was counted by double layer agar method. The effects were checked twice.

Storage stability of bacteriophages

Storage stability means that bacteriophages are stored under different conditions during their usage e.g. room temperature 4C and -20C etc. So we have to see that our phage can survive under these conditions for longer time say two or three months or not. For that purpose 500 ulof 10^9 S.A in three Eppendorf and one was kept at room temperature second was kept in freezer and third was kept in refrigerator for two months and after two months number of viable phages was evaluated by double agar overlay method(Capra, Quiberoni *et al*., 2006).

RESULTS

Host bacterial strains

Two host bacterial strains of *staphaureus* were streaked and incubated at 37 C for 24 hr these bacterial strains served as host against the isolated bacteriophage in all experiments.

Host range determination for bacteriophage

The host range of isolated phage was check for different bacterial strains e.g., *staph aureus* and some probiotics. The host range determines whether or not a single type of bacteriophage can lyse more than one type of bacteria. This depicts the use of bacteriophage at different level for phage therapy. Phage do not showed lytic activity for any bacterial strains other than its own host which showed the narrow host range.

Phage count and plaque morphology

Plaque forming unit per milliliter (PFU/ml) were calculated along with the observation of plaque morphology were recorded. The plaque morphology of phage was small and

circular on double agar layer plates. The size of the plaques varied from 1-5mm the plaque count of isolated phages ranged between $10^9 – 10^{12}$.

Picture 3.2. Plaques of *Staph aureus:* PFU/ml was calculated by double layer agar method was $10^9 – 10^{12}$

Plaque Enrichment

The plated plaques were observed for any increase in size after incubation of 24, 48 and 72 hour at 37 C. the plaque enhancement signifies the potential of bacteriophages to replicate and lyse the host bacteria with in the same condition even after the usage of the nutrients and completion of host bacterial rapid growth the phage shows no enhancement after 48 hour as compared to 24 hour incubated plaque size.

Physiological characterization of Bacteriophages

Effect of pH on stability of bacteriophage

The effect of different pH on the viability of isolated phage is important to check so that preservation, usage and application of this phage can properly be handled through phage therapy. It was observed by treating the bacteriophage at different pH for 1 hour and PFU/ml was determined.PFU/ml was maximum at 7 pH and minimum at 3 pH and 9, 10 pH.No phages seen at 12 pH.

Table PFU/ml was maximum at seven pH.*staph aureus* phage was so stable to wide range of pH and there was not any significant decrease in the phage titer even at 3 and 9 pH values.

Sr #	pH	Phages
1	3	2.5×10^2
2	5	3.2×10^5
3	7	4.1×10^8
4	9	3.6×10^6
5	12	Nil

Effect of temperature on stability of bacteriophage

The thermal stability of phages is significantly of use to help standardize the phage therapy technique and keep phages working even through harsh conditions. Thermal stability of isolated phages was checked after calculating the PFU/ml through the appeared plaque count. The PFU was highest at 37 C and few plaques were observed at all temperature used for this treatment in this experiment. No plaques were observed at 105 C.

Table 3.2. Thermal stability of isolated phages was checked after incubating at corresponding temperature and then determining phage titer by phage titer determination method.Phage S.A was so resistant to extreme temperature even at 100ºC for overnight incubation in oven and the phage titer was maximum at 37°C.

Serial No	Temperature	Phages
1	30 ^{0}C	1.4×10^9
2	37 ^{0}C	3.4×10^{10}
3	50 ^{0}C	5.1×10^8
4	70 ^{0}C	6.3×10^7
5	80 ^{0}C	5.2×10^5
6	100 ^{0}C	6.1×10^2
7	105 ^{0}C	Nil

DISCUSSION

Mastitis has been one of the costly disease of dairy animals and is responsible for significant lose in term of animals as well as money. Many pathogens have been found to be associated with causing this disease but staphylococcus aureus considered one of the most important pathogen causing mastitis.Because the pathogenic potential of staphylococcus is very much aided by its ability to have resistance to antibiotics. So a promising alternative treatment against bovine mastitis is in the form of phage therapy(Han, Kim *et al*. 2013) (Kwiatek, Parasion *et al*. 2012) (Garcia, Madera *et al*. 2009). It has already been used against many pathogens such as Ecoli(Dąbrowska, Skaradziński *et al*. 2010) (Matsuzaki, Yasuda *et al*. 2003).. (Capparelli, Parlato *et al*. 2007), (Dąbrowska, Skaradziński *et al*. 2010; Kwiatek, Parasion *et al*. 2012). In current study we have characterized phages against staphylococcus aureus causing mastitis. Phages have lytic activity against pathogenic staphylococcus aureus which were isolated from milk of infected cow. After isolation and purification of phages their host range was determined and for this purpose phages were allowed to grow on different bacterial strains and their ability to infect those bacteria was determined by observing the formation of plaques in case of each new hosts.

It was found that our isolated phages were having narrow host range due to their ability to specifically infect only one bacterial strain which was *Staphylococcus aureus.*This phage was produced at large scale for further experimental work.Physiological characterization of isolated phages was also done for this purpose it affects of different environmental factors was checked on the ability of the phages to make plaques. First of all effect of pH was determined for this purpose phages were exposed to different pH and their ability to make plaques was determined it was found maximum PFU/ml were seen at pH 7 and they were around 4.1×10^8. Decrease in pH cause reduction in the number of PFU and it was seen that it reduced to 3.2×10^5 at pH 5 and 2.5×10^2 at ph 3. So decrease in pfu/ml was seen with decrease in the ph. By increasing pH same results were seen because increase in pH above 7 reduced the number of pfu/ml. At pH 9 there were 3.6×10^6 pfu/ ml and when ph was further increased to 12 there was marked decrease in the reduction of the pfu/ml because no plaques were seen at this pH.Along with the effect of pH effect of temperature was also checked on the stability of phages. At 37 C maximum pfu were observed which were 3.4×10^{10} pfu/ml. change in temperature also had effect on the stability of the phages as we decrease temperature upto 30 C it decreased the pfuupto 1.4×10^9 and same was in the case of increase in

the temperature. Increase in the temperature more than 37 C cause the reduction in PFU/ml and it reduced to 5.1×10^8 at 50 C, 6.3×10^7 at 70 C and 5.2×10^5 at 80C. Marked decrease in PFU was seen when temperature was raised to 100C and it was reduced to 6.1×10^2. When temperature was further increased to 105 C there were no plaques seen at that temperature. This effect of environmental factor was determined because it has importance in the overall activity of the phages. End purpose of this study was to use phages in phage therapy and for this purpose phages must be stored at appropriate conditions. So appropriate temperature and ph for the storage and best activity of the phages has great importance and has direct effect on the use of phages in the phage therapy.

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