ejpmr, 2016,3(4), 412-417

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 3294-3211

EJPMR

CHARACTERIZATION OF BACTERIOPHAGE AGAINST *STAPHYLOCOCCUS AUREUS CAUSING* MASTITIS

Shafeeq-ur-Rehman[1], Taimoor Khan[2], Farhan Rasheed[1] and Shahid Raza[2]*

[1]University of Punjab, Lahore, Pakistan.
[2]University of South Asia, Lahore Pakistan.

***Corresponding Author: Dr. Shahid Raza**
University of South Asia, Lahore Pakistan.

Article Received on 22/02/2016 **Article Revised on 14/03/2016** **Article Accepted on 05/04/2016**

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 characterized bacteriophage against *staphylococcus aureus* causing mastitis which is isolated from the sewage water of dairy farm. The bacteriophage showed lytic activity against these bacteria. The phage efficiently reduced bacterial growth in the bacterial reduction assay. So it signifies the underlying potential of bacteriophage therapy.

KEYWORDS: *staphylococcus aureus*, bacteriophage therapy.

INTRODUCTION

The most common reason of death in fully developed dairy cows is Mastitis which is a inflammation of parenchyma of mammary glands.(Sudhan and Sharma 2010). It is measured the most regular production disease in the developing countries. (Rajala-Schultz, Gröhn et al. 1999; Seegers, Fourichon et al. 2003) This disease costs the loss of 35$ billion US dollars per year. Staphylococcal Mastitis can be especially manifested clinical and subclinical infection that keeps hold 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.To remove bacteria including staphylococcus in human infection, bacteriophages were researched as antibacterial agents in 1920 and the results 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).

MATERIAL

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

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

Table 2.6 10 X TAE (Tris Base- Glacial acetic acid -EDTA)

S. No.		Components	L^{-1}
1.	**2.**	Tris base	108.0gm
3.	**4.**	Glacial acetic acid	55.0 gm
5.	**6.**	Sodium EDTA	9.25 gm

Table 2.7 1X TAE Running Buffer (Working solution)

S. No.		Components	L^{-1}
1.	**2.**	10X TAE	50
3.	**4.**	Distilled water	950

Table 2.8 5X loading dye

S. No.		Components	L^{-1}
1.	**2.**	Bromophenol blue	25.0 mg
3.	**4.**	Glycerol	3 ml
5.	**6.**	H_2O	7.0 ml

Mix glycerol and bromophenol blue. Add water and store at 4^0C.

Table 2.9 1% agarose gel

S. No.	Components	L^{-1}
1	Agarose 1gm (1%)	1 gm (1%)
2	1X TAE	100 ml

METHOD

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.

One Step Growth Curve of Bacteriophages: The one step growth bend was executed in doubles as stated by effectively reported strategy (Henle, Henle et al. 1947) with a few alterations. The host strain was incubated at 37°C to the mid-exponential stage with $O.D_{600}$ as 0.4-0.6 and the cells were collected by centrifugation. The pellet was re-suspended in 500ul of L-broth. The filtered bacteriophages were supplemented with the bacterial re-suspension and incubated at 37°C for one moment. The mixture was centrifuged at 13000 rpm for 30 seconds to kill free phages. At that point the pellet was re-suspended in 100 ml L-broth and the culture was incubated at 37°C consistently. Sample was taken at 5 minute interval for 60 minutes, centrifuged and the phage titer was assessed by a double layer agar method.

The Bacterial Growth Reduction Assay

The 24 hours fresh host culture was added to two L-broth flasks. One flask was inoculated with 1 ml of respective phage by keeping. The other one was taken as control containing no phages. The flasks were incubated on a shaking incubator at 120 rpm at 37°C. The optical

density (0.D 600 nm) taken at an interval of 2 hours for 24 hours (Ul Haq, Chaudhry et al. 2012).

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 ul of 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).

DNA isolation: Bacteriophage DNA was isolated by following phage hunting protocol. In this protocol, 1 ml of phage lysate and 12.5µl of 1M $MgCl_2$ were transferred to a 15 ml conical tube and then mixed it gently. After mixing gently, 0.4 µl DNAse I (2000 U/mL) and 1 µl RNAse A (100 mg/mL) were added to lysate-$MgCl_2$ mixture and vortexes briefly. The mixture was incubated at room temperature for 4 hours. After incubation, 40 µl of 0.5 M EDTA, 5 µl of Proteinase K (10 mg/mL) and50 µl of 10% SDS were added.

Then the mixture was Vortex vigorously and Incubated at 55°C for 60 minutes. Mixture was Vortex vigorously twice during incubation, at 20-minute intervals. Two 1.5 ml micro centrifuge tubes were taken. Transferred 500 µl of the mixture to each tube and all materials were transferred to a chemical fume hood. Equal amount of PCI (500 µl for the first time) was transferred to each tube with 500 µl of lysate. Invert tubes several times to mixed well. Centrifugation was done for 5 minutes at room temperature at 13K rpm then the top aqueous layer was removed above the white interphase.

1 ml of 95% ethanol and 50 µl of 3M sodium acetate solution were added to the aqueous layer. Sample was placed on ice for 5 minutes. Mixed gently, and the DNA will form a "cotton ball" like precipitate. Centrifugation was done at room temperature for 10 minutes at 13K rpm. Place the cap fold to the outside as an indicator to where the pellet would be. Decant the tubes carefully.

Then 500 µl of 70% ethanol was added to wash the pellet. Simply let ethanol was run through the pellet. Centrifugation was done for 10 minutes at 13K rpm at room temperature. Decant the tubes, and carefully pipet out any remaining droplets. Air dried the pellet ~10 – 20 minutes. DNA was dissolved in ~50 µl dis.H_2O. To ensured complete solvation, the tubes were set in 37°C for 10 minutes. DNA was stored at 4°C for the short term. For long-term storage, store at –20°C.

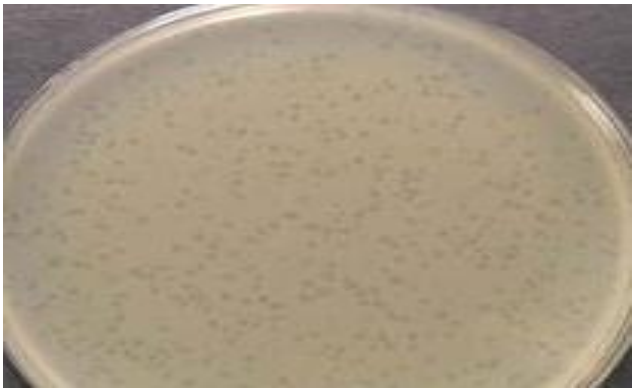
2.6 Gel electrophoresis: Gel electrophoresis was done to visualize DNA in our sample. For that purpose 1% agarose gel was made in 1X TAE buffer. Ethidium bromide is added to the gel because it is an intercalating agent and DNA bands glow under U.V light in the presence of Ethidium bromide. DNA samples was loaded in the wells along with ladder after mixing with the loading dye bromophenol blue loading dye in proportion of 4:1(Table 2.6 C). DNA is negatively charged so gel was run from negative to positive direction in the gel apparatus i.e. from black to red. After that gel was observed under U.V illuminator.

RESULT

Host bacterial strains: Two host bacterial strains of *staph aureus* 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.

3.4.1 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 $10^9 – 10^{12}$

3.5.4 One step growth curve of bacteriophages: One step growth curve has importance in verifying the latent period and burst size of the bacteriophages that detects the level of lytic ability of the phages. The shorter latent period with higher burst size may be a valuable add-up to phage therapy research. The sample were taken after every 5 minute interval and plated. The sampling was carried on for an hour and the plates were incubated for 24 hours at 37 C. the resultant plaques were counted and tabulated and latent period and burst size was calculated.

Table 3.4 One step growth curve has an importance in verifying the latent period and burst size of the bacteriophage S.A that detects the level of lytic ability of the phages. The sampling was carried on for an hour and the plates were incubated for 24 hours at 37 C. the resultant plaques were counted and tabulated and latent period and burst size was calculated.

Serial No	Time (min)	Phages (PFU/ml)
1	5	3.6×10^4
2	10	4.4×10^4
3	15	6.5×10^4
4	20	2×10^2
5	25	2×10^2
6	30	6.7×10^5
7	35	8.8×10^7
8	40	9.8×10^8
9	45	1×10^9
10	50	5×10^8
11	55	6×10^9
12	60	7×10^9

3.5.5 The bacterial growth reduction assay

The bacterial growth reduction assay holds a tremendous relation towards phage therapy as it indicates the reduction of bacterial growth through incubation time with bacteriophages. The maintained bacterial reduction is actually of great interest in the phage therapy. The reduction in the growth of the host bacterium was observed for the phage, compared with the non-infected host culture which served as control. O.D was taken at 600nm.

Table 3.5 The reduction in the growth of the host bacterium was observed for the phage, compared with the non-infected host culture which served as control. O.D was taken at 600nm.

Serial No	Time (min)	O.D. at 600 nm	
		Control	With phage
1	2	0.045	0.002
2	4	0.100	0.006
3	6	0.236	0.015
4	8	0.268	0.046
5	10	0.378	0.091
6	12	0.496	0.167
7	14	0.679	0.351
8	16	0.812	0.462
9	18	0.900	0.568
10	20	1.000	0.816
11	22	1.225	1.001
12	24	1.432	1.322

3.5.6 Genomic DNA

The genome of the bacteriophage was isolated and observed under UV on 1 % agarose gel after running it for one and half hour. The genome of bacteriophages appeared above the 1 Kb plus ladder that has maximum size of 1kb. It shows that the genome of isolated bacteriophages is surprisingly greater than 1 kb. The band seen on the agarose gel was not distinct rather a smear was formed which may indicate that isolated DNA was degraded or had some contamination.

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 effects of different environmental factors was checked on the ability of the phages to make plaques.

To check the latent period of the bacteriophage one step growth curve was done. It is important in the estimation of the burst size and burst period of the bacteriophage because it has great importance in the phage therapy. The shorter latent period with higher burst size may be a valuable add-up to phage therapy research. The sample were taken after every 5 minute interval and plated. Our results indicates that burst period of our phage was around 30 mints because before 30 minutes PFU/ml were comparatively less and after 30 mints they started increasing rapidly. This clearly indicates that 30 mints is the burst period of our bacteriophage.

Bacterial growth reduction assay was performed. It is important test which provide us information about bacterial growth reduction in the presence of the phage when incubated together and it is important in the process of phage therapy because it directly indicate ability of our bacteriophage to attack and kill their hosts. Our results indicate that growth reduction in the form of optical density was seen in the presence of phage as compared to the control which was without phages. This reduction assay was done from 2 mints to 24 mints and optical densities at these time intervals clearly indicate the ability of our phage to kill their hosts.

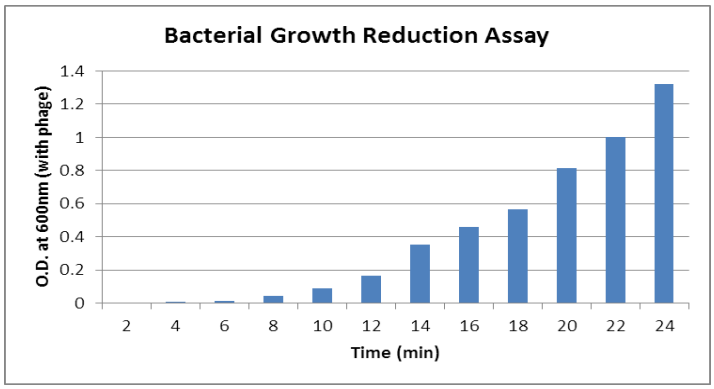


Figure 4.6 Bacterial Growth Reduction Assay.

Reduction assay was done from 2 mints to 24 mints and optical densities at these time intervals clearly indicate the ability of our phage to kill their hosts.

These factors were 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.

REFERENCE

1. Capparelli, R., M. Parlato, et al. "Experimental phage therapy against Staphylococcus aureus in mice." Antimicrobial agents and chemotherapy, 2007; 51(8): 2765-2773.
2. Capra, M. L., A. Quiberoni, et al. "Phages of Lactobacillus casei/paracasei: response to environmental factors and interaction with collection and commercial strains." J Appl Microbiol, 2006; 100(2): 334-342.
3. Dąbrowska, K., G. Skaradziński, et al. "The effects of staphylococcal bacteriophage lysates on cancer cells in vitro." Clinical and experimental medicine, 2010; 10(1): 81-85.
4. Dixon, B. "New dawn for phage therapy." The Lancet infectious diseases, 2004; 4(3): 186.
5. Garcia, P., C. Madera, et al. "Prevalence of bacteriophages infecting Staphylococcus aureus in dairy samples and their potential as biocontrol agents." Journal of dairy science 2009; 92(7): 3019-3026.
6. Green, M. and A. Bradley. "Clinical Forum—Staphylococcus aureus mastitis in cattle." Cattle pract, 2004; 9: 1-9.
7. Han, J. E., J. H. Kim, et al. "Isolation and characterization of a Myoviridae bacteriophage

against Staphylococcus aureus isolated from dairy cows with mastitis." Research in veterinary science, 2013; 95(2): 758-763.
8. Harper, D. and M. Enright. "Bacteriophages for the treatment of Pseudomonas aeruginosa infections." Journal of applied microbiology, 2011; 111(1): 1-7.
9. Henle, W., G. Henle, et al. "The demonstration of one-step growth curves of influenza viruses through the blocking effect of irradiated virus on further infection." The Journal of experimental medicine, 1947; 86(5): 423-437.
10. Kwiatek, M., S. Parasion, et al. "Characterization of a bacteriophage, isolated from a cow with mastitis, that is lytic against Staphylococcus aureus strains." Archives of virology, 2012; 157(2): 225-234.
11. Levin, B. R. and J. J. Bull. "Population and evolutionary dynamics of phage therapy." Nature Reviews Microbiology, 2004; 2(2): 166-173.
12. Levy, S. B. and B. Marshall. "Antibacterial resistance worldwide: causes, challenges and responses." Nature medicine, 2004; 10: S122-S129.
13. Li, L. and Z. Zhang. "Isolation and characterization of a virulent bacteriophage SPW specific for Staphylococcus aureus isolated from bovine mastitis of lactating dairy cattle." Molecular biology reports, 2014; 41(9): 5829-5838.
14. Matsuzaki, S., M. Yasuda, et al. "Experimental protection of mice against lethal
15. Staphylococcus aureus infection by novel bacteriophage ϕMR11." Journal of Infectious Diseases, 2003; 187(4): 613-624.
16. Rajala-Schultz, P., Y. Gröhn, et al. "Effects of clinical mastitis on milk yield in dairy cows." Journal of dairy science, 1999; 82(6): 1213-1220.
17. Rose, H. "My enemy's enemy is, only perhaps, my friend." Social Text, 1996: 61-80.
18. Seegers, H., C. Fourichon, et al. "Production effects related to mastitis and mastitis economics in dairy cattle herds." Veterinary research, 2003; 34(5): 475-491.
19. Smith, T. L., M. L. Pearson, et al. "Emergence of vancomycin resistance in Staphylococcus aureus." New England Journal of Medicine 1999; 340(7): 493-501.
20. Sudhan, N. and N. Sharma "Mastitis-An Important Production Disease of Dairy Animals." SMVS Dairy Year Book, 2010: 72-88.
21. Sulakvelidze, A., Z. Alavidze, et al. "Bacteriophage therapy." Antimicrobial agents and chemotherapy, 2001; 45(3): 649-659.
22. Thacker, P. D. "Set a microbe to kill a microbe." JAMA, 2003; 290(24): 3183-3185.
23. Thiel, K. "Old dogma, new tricks--21st Century phage therapy." Nature biotechnology 2004; 22(1): 31-36.
24. Ul Haq, I., W. N. Chaudhry, et al. "Isolation and partial characterization of a virulent bacteriophage IHQ1 specific for Aeromonas punctata from stream water." Microb Ecol 2012; 63(4): 954-963.
25. Yang, H., L. Liang, et al. "Isolation and characterization of a virulent bacteriophage AB1 of Acinetobacter baumannii." BMC microbiology, 2010; 10(1): 1.