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Isolation and Identification of Novel *Staphylococcus* Phage as a Novel Therapeutic Agent from Sewage of Livestock Farms

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ABSTRACT

Background: Bacteriophages are viruses that infect bacteria. They are obligate intracellular infective agents requiring specific bacterial organism as a host cell for their replication. They are most widely distributed and diverse entities in the biosphere and ubiquitously present in all reservoirs populated by bacterial hosts, such as soil and the intestine of animals. Bacteriophages are very specific in nature selectively replicate in particular bacteria.

Methods: In present study we have isolated the bacteriophages from sewage of various livestock farms. Sewage samples (n=150) were collected from different depths of the sewage collection tank from different species *viz*. cow, buffalo, goat and pig. All the samples were subjected primarily to isolation bacteriophage against *S. aureus*. Recovered bacteriophages were identified with transmission electron microscopy (TM).

Result: Total of twenty seven sewage samples were showing plaque formation by producing lytic activity against *S. aureus* in double agar overlay method. The recovery percentage of bacteriophages was maximum from cattle farm sewage (30%), followed by buffalo farm sewage (20%) and pig farm sewage (17.50%). The result indicated that recovered Bacteriophage had icosahedral symmetry and head size 52.20 nm with 109 nm tail. Bacteriophage selectively infected *S. aureus* with a narrow host range. Results indicated that the recovered isolates of bacteriophage remained viable at 50°C for one and two min and no viable survivors were seen at the 5 min exposure. They remained viable at 60°C for 1 min only and did not show viability for 2 and 5 min exposure at 60°C. None of the bacteriophages survived at 70°C for 1, 2 and 5 min. Bacteriophage isolates were completely inactivated below pH 3 and above 11; they remained viable only in pH range of 5 to 9.

Key words: Bacteriophage, DAL, Lysate, Myoviridae, Recovery, Sewage.

INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium, also known as the ´golden staph´ due to the characteristic yellow pigment production. *S. aureus* can cause serious infections in both humans and animals with fatal consequences as well as food poisoning (Quinn, 2011). This bacterium is common and widespread, being present on the skin and in the nose of healthy people as a commensal. *S. aureus* can cause infections with severe outcomes ranging from pustules to sepsis and death (Kazmierczak *et al.,* 2014). *S. aureus* is commonly associated with mastitis and having multiple drug resistant (Shrivastava *et al.,* 2017).

Due to its high resistance, alternative antimicrobial strategies are under development. Use the *Staphylococcus phage* is seen as an important strategy to combat pathogenic organisms (Melo *et al.,* 2014). *S. aureus* phages have the capability of eliciting efûcient lysis of *S. aureus*, revealing it potentially as an effective approach to prophylaxis or treatment of *S. aureus*-associated mastitis in dairy cows (Li and Zhang, 2014). Phage lysates having very good efficacy against the septic wounds infections associated with *S. aureus* (Shukla *et al.,* 2021).

Bacteriophages used against MRSA are a new, potential alternate agent. They are the most abundant microorganism on earth and coexist with the bacterial population. They can destroy bacterial cells successfully and effectively. They cannot enter mammalian cells which save the eukaryotic cells from lytic activity and have no side effect on cells (Nasser *et al.,* 2019).

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Phages are the most widely distributed and diverse entities in the biosphere and ubiquitously present, which can be found in all reservoirs populated by bacterial hosts, such as soil and intestine of animals (McGrath and van Sinderen, 2007). Bacteriophages were co-discovered by Twort and d'Herelle. *S. aureus* phages are DNA viruses of Myoviridae family (Ackermann, 2009) come under the order Caudovirales (Xia and Wolz, 2014). They are natural predators of bacteria and are found ubiquitously; these organisms are estimated to be present at numbers equivalent to a trillion per grain of sand on earth (Keen, 2015). The main aim of this study was to isolate and characterized the *S. aureus* phage from sewage samples of various livestock farms.

MATERIALS AND METHODS

The present research work was performed during the year 2018-19 (12 months period) in the Department of Veterinary Microbiology and Livestock farms, Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur (M.P.), India. Culture media like basal agar (tryptone broth with 1.5% agar), soft agar (tryptone broth with 0.7% agar), mannitol salt agar, nutrient agar and nutrient broth were used during the study.

Sewage samples for bacteriophage isolation

Sewage samples for bacteriophage isolation were collected from Livestock Farms (cattle, buffalo, pig and goat), NDVSU, Jabalpur (M.P.). Sewage material consisted of various body excretions from different species of animal's *viz.* cattle, buffalo, goat and pig. There were 150 sewage samples collected in test tubes. A pipette, fitted with a rubber bulb was used to aspirate the sewage fluid (50 ml) from the various collection tanks and storage pits.

Isolation of bacteriophage from sewage of livestock farms

Isolation of bacteriophages against *S. aureus (*ATCC 25923) from sewage samples was done by soft agar overlay method as described by Synnot *et al.* (2009) with slight modification. The collected sewage samples were centrifuged at 6000 rpm for 10 min. The supernatant of centrifuged sewage samples were filtered through 0.45 µm syringe filters. The filtrates of sewage samples (15 ml) were mixed with *S. aureus* culture (10 ml) of log phase and 25 ml of tryptone soya broth and incubated at 37°C overnight. The mixture was centrifuged and filtered through 0.22 µm syringe filter. A 100 µl of bacterial culture and 50µl of phage filtrate were incubated for 20 min and added to 3 ml of molten soft agar, spread tryptone soya agar plates and incubated at 37°C for 18 h to observe the formation of plaques.

Elution of *Staphylococcus* bacteriophage from the plaques

Elution of bacteriophage from the plates of plaques was done according to Sangha *et al.* (2014) with slight modification. The double agar overlay plates with plaques were selected for the purification of recovered bacteriophage. Salt of magnesium (SM) buffer was poured on the bacteriophage grown plates. The entire surface of semisolid material was scrapped and scrapping material with SM buffer was collected in centrifuge tubes. The phage lysate suspension was centrifuged at 6000 rpm for 5 min at 4°C. The supernatant was filtered through 0.22 µm syringe filter and stored at 4°C for further use.

Characterization of recovered isolates of bacteriophages

Recovered bacteriophages were subjected for characterization by various ways like plaque morphology, transmission electron microscopy, host range determination, the effect of temperature and pH.

Morphology of plaques was described by the method of Ellis and Winters (1969). Recovered bacteriophages isolates were characterized according to the size of the plaque (small sized and large sized) and boundaries of plaque (clear or diffused). Structural identification of the bacteriophage was done by electron microscopy as described by Owens *et al.* (2013) with slight modification. A 6 µl of a concentrated phage suspension (10^{10} PFU/ml) in SM buffer was spotted on top of a hydrophilic carbon-coated copper grid and the sample was allowed to adsorb for 2 min and then stained by aqueous phosphor-tungstic acid and visualized under TEM.

Host range of phage isolates was determined by using the method of Goodridge *et al.* (2003) with slight modification. To observe the lytic activity of recovered bacteriophages isolates against other pathogenic bacterial organisms.

Effect of pH (ranging from 3 to11) was seen on phage titer and viability of phages in broth was observed using the method of Grilione and Carr (1959). Bacteriophage lysate mixed in broth having different pH levels (3-11) and incubated for one hour at 37°C. Similarly, to study the effect of different temperatures on bacteriophages viability were incubated at different temperatures (50°C and 60°C and 70°C) for 1, 2 and 5 min in broth (pH 7.0) by using the method of Lu *et al.* (2003). The incubated phage was independently mixed with exponential growth culture of the host bacteria and observed the formation of plaques.

RESULTS AND DISCUSSION

Isolation of phage from sewage samples of livestock farms

A total of 150 sewage samples, collected from various livestock farms were processed for isolation for phage. These collected sewage samples were subjected to the isolation of bacteriophage by double agar layer method. A systematic approach was followed for the isolation of phage using *S. aureus* as a host bacterium (Fig 1). Presences of bacteriophage in samples were detected by the formation

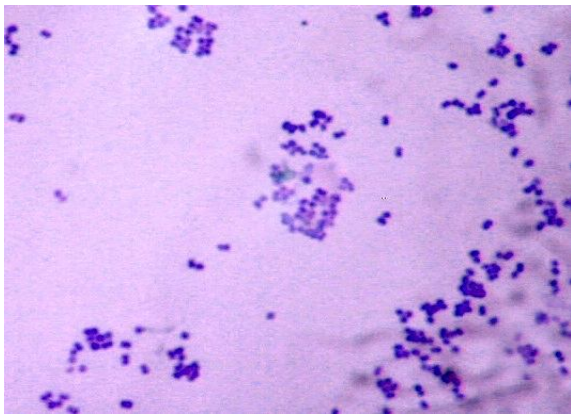

Fig 1: *S. aureus* as a host bacterium (100x) for bacteriophage isolation.

of clear plaque on the agar plates. The plates which were showing the plaque formation were selected for the characterization. A similar approach was also adopted by Synnott *et al.* (2009).

A total of 27 samples (18%) were showing the lytic activity against the *S. aureus* for the isolation of bacteriophage out of one fifty sewage sample. Twelve sewage samples were positive from cattle farm followed by eight samples positive from buffalo farm and minimum seven samples were positive from pig farm sewage samples. Goat farm samples were not showing any lytic activity against the *S. aureus* (Table 1).

Characterization of staphylococcal phages

Plaque morphology of isolated bacteriophage

The recovered isolates of *Staphylococcus* bacteriophages were firstly subjected for characterization on the basis of their plaque morphology. The first and more important identification of the *Staphylococcus* phages was done by observing the plaques over the double layer agar plates. Plaques were categorized into small and large sizes with diffused and clear boundaries (Table 2). Similar approach was adopted for characterization of bacteriophages by Ackermann (2003) and Deghorain and Van Melderen (2012).

In our study, a total of twenty seven sewage samples showed plaque formation by producing lytic activity against *S. aureus* in double agar overlay method out of 150 sewage samples. Out of twenty seven lytic samples, nine sowed clear plaque and rest eighteen showed diffused plaques (Fig 2).

Transmission electron microscopy

In the present study electron microscopy was done to identify and characterized the bacteriophage isolated from sewage samples from various livestock farms of ILFC, NDVSU, Jabalpur to confirm the morphological structures. Bacteriophages were caudate and belonged to the myoviridae family. A similar approach was adopted by Kumari *et al.* (2009); Synnott *et al.* (2009) and Owens *et al.* (2013).

The recovered isolates of bacteriophages with clear plaque were selected for transmission electron microscopy for morphological studies. Transmission electron microscopy showed the recovered bacteriophages had hexagonal structure with tail fiber. TEM revealed that the bacteriophage had an icosahedral symmetry with the head size 52.20 nm in diameter and long tail of 109 nm. Head and tail were held together by a connector of 15-18 nm long and can be classiûed as a member of the Myoviridae family under the order of Caudovirale (Fig 3). Similar approach was adopted by a number of workers for the characterization of phages. Our findings were in agreement with many previous worker like Ackermann *et al.* (2007); Dias *et al.* (2013); Li and Zhang (2014); Kazmierczak *et al.* (2014) and Mohammed-Ali and Jamalludeen (2015).

Host range determination

Although bacteriophages are very specific to the particular host means they specifically infect selected genera. The recovered bacteriophage Isolates were subjected to determination of host range of their lysate in terms to infect the other bacterial organisms like *Bacillus* spp, *Salmonella*

Table 1: Number of bacteriophage isolated against *S. aureus* by DAL method.

Source of samples	Number of Isolated phage	Percent recovery
Cattle farm	12(40)	30.00%
Buffalo farm	08(40)	20.00%
Pig farm	07(40)	17.50%
Goat farm	00(30)	00%
Total	27(150)	18%

Table 2: Characterization of phage on the basis of plaque morphology.

Plaque morphology of phage	Name of phage	Number of phage
Small sized clear plaque	ØVS^{scp}	05
Large sized clear plaque	ØVS^{lcp}	04
Small sized diffused plaque	ØVS^{sdp}	12
Large sized diffused plaque	ØVS^{ldp}	06
Total		27

Fig 2: Bacteriophage plaques on tryptone soya agar plate.

spp, *E. coli* and *Pseudomonas* spp. Similar approach was adopted by Goodridge *et al*. (2003) to determine the host range of phage.

Most of the lysate of bacteriophage were showing lytic activity specifically against the *S. aureus*. They had shown a very limited host range. Three isolates (ØVS4 ØVS5 and ØVS9) of phage lysate had shown the lytic activity against the *Bacillus* spp and *E. coli.* These phage lysates were also showing lytic activity against other microorganisms indicated that they had a broad host range. These findings further correlate to the earlier reports of Bielke *et al.* (2007) who reported that phage host range is not always genera restricted and phages could be of wide host range (Table 3).

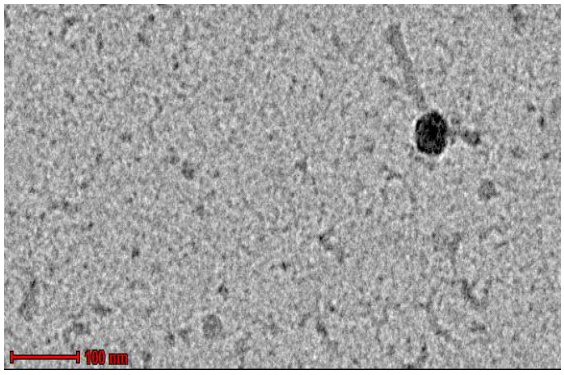

Fig 3: Transmission electron microscopic image of *S. aureus* phage (100 nm).

Wide range of pH and temperature

Recovered bacteriophages (27) were studied to observe the effect pH and temperature on their viability at various temperatures and time intervals. Similar approaches were adopted by Grilione and Carr (1959) and Lu *et al.* (2003).

Regarding the effect of pH, all bacteriophage isolates showed viability ranging from pH 5 to 9 in LB broth. Observations on pH sensitivity indicated, no significant effect of pH on the viability of phage isolates which appeared to be stable at pH range 5 to 9 but inactivation was evident at the very low (pH 3) and very high pH levels (pH 11). These findings were in complete confirmation with earlier reports by Sharp (2001), Jamalludeen *et al.* (2007); Dias *et al.* (2013); Mohammed-Ali and Jamalludeen (2015) and Hamza *et al.* (2016) who described that phage viability was maximal between pH 5 and 9 and all phages were completely inactivated at pH values of 3 and 11 (Table 4).

Similarly, to study the effect of different temperatures on bacteriophages, they were incubated at different temperatures (50°C and 60°C and 70°C) for 1, 2 and 5 min in tryptone soya broth (pH 7.0). This was conducted to observe the thermal tolerance of phage. The incubated bacteriophage was independently mixed with exponential growth culture of the host bacteria and the numbers of plaques were counted by the double layer agar method (Adams, 1959).

Results indicated that the recovered isolates of bacteriophage remained viable at 50°C at 1 min and 2 min

Table 3: Characterization of bacteriophage on the basis of host range.

Plaque morphology	Phage tested	No. of isolates	Host range			
			S. aureus	*E. coli*	*P. aeruginosa*	*Bacillus subtilis*
Small sized, clear plaque	$ØVS^{scp}$	05	05	02	0	02
Large sized clear plaque	$ØVS^{lcp}$	04	04	00	0	00
Small sized diffused plaque	$ØVS^{sdp}$	12	12	01	0	01
Large sized diffused plaque	$ØVS^{ldp}$	08	08	00	0	00

Table 4: Viability of phage lysate at various pH levels.

Phage tested	No. of isolates	Viability of phage lysate at different pH				
		3	5	7	9	11
$ØVS^{scp}$	05	0	05	05	05	0
$ØVS^{lcp}$	04	0	04	04	04	0
$ØVS^{sdp}$	12	0	12	12	12	0
$ØVS^{ldp}$	08	0	08	08	08	0

Table 5: Viability of phage lysate at different temperature.

Bacteriophage	No. of isolates	Viability at 50°C			Viability at 60°C			Viability at 70°C		
		1 min	2 min	5 min	1 min	2 min	5 min	1 min	2 min	5 min
$ØVS^{scp}$	05	05	05	00	05	00	00	00	00	00
$ØVS^{lcp}$	04	04	04	00	04	00	00	00	00	00
$ØVS^{sdp}$	12	12	12	00	12	00	00	00	00	00
$ØVS^{ldp}$	08	08	08	00	08	00	00	00	00	00

and no viable survivors were seen at the 5 min exposure. They were viable at 60°C for 1 min only and did not show any viability for 2 and 5 min with 60°C. In our study none of the bacteriophage survived at 70°C for 1, 2 and 5 min (Table 5)

These findings are in conformity with those reported by Lu *et al*. (2003) who reported that bacteriophage get inactivated at 70°C and above. The reduction of burst size at higher temperature is probably the result of the effect of higher temperature on the metabolism of the host, because the bacterial growth rate is decreased between 45°C and 51°C (Nishihara and Romig, 1964).

CONCLUSION

Isolation of bacteriophage was recorded maximum for cattle farm sewage (30%) followed by buffalo farm sewage (20%) and pig farm sewage (17.50%). The bacteriophages were not recovered from goat farm sewage. Prevalence of bacteriophage was maximum from deep layer of cattle sewage as compared to superficial layer. Recovered bacteriophages had hexagonal structure with tail fiber. Bacteriophage had an icosahedral symmetry with the head size 52.20 nm in diameter and long tail of 109 nm and they were classiûed as a member of the Myoviridae family under the order of Caudovirale. Most of phages recovered from sewage had a very narrow host range specifically infecting S. *aureus*. Bacteriophages were viable at the temperature of 50°C and pH range of 5-9.

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