Int.J.Curr.Microbiol.App.Sci (2021) *10*(03): 1300-1306

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 10 Number 03 (2021)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2021.1003.158

Isolation and Partial Characterisation of *Streptococcus suis* Bacteriophage

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Keywords

Streptococcus suis, Bacteriophage, Antibiotic resistance, PFU

Article Info

Accepted:
12 February 2021
Available Online:
10 March 2021

A B S T R A C T

Streptococcus suis serotype 2 infection is considered as a major pathogenic disease worldwide both in humans and swine. In the present study, isolation of lytic phages against *S. suis* serotype 2 would provide therapeutic potential in combating the antibiotic resistant strains of *S. suis* organisms. Four lytic bacteriophages SS1, SS2, SS3 and SS4 were recovered from the sewage samples at piggery farm. Upon host range determination, SS1, SS2, SS3 phages were found to be lytic against *S. suis* serotypes 1/2, 2, 3, 7 and 9 where as SS4 phage was found to be lytic against only serotype 2 and 7. SS1, SS2 and SS3 phages were found to be lytic against other gram positive organisms like Methicillin Resistant *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus dysgalactiae* and *Enterococcus* sp. indicating broader lytic spectrum. Three different phage morphotypes were observed against *S. suis*. SS2 phage was having the larger plaque size of 4mm in diameter in comparison to other phages with highest mean phage titre of 2×10^{14}PFU/ml i.e., 14 log PFU count and hence SS2 phage was subjected for temperature, pH and chemical stability testing. SS2 Phage was found to be stable on incubation for 30min at 4^{o}C and 20^{o} C, showing highest activity at 4^{o}C with count of 2×10^{14} PFU/ml. Phage titre was dropped to 4×10^{4} PFU/ml at 60 ^{o}C. Upon pH stability, the SS2 phage was having stable phage counts at pH 2, 7 and 11 whereas highest lytic activity was observed at pH 7 with counts of 2×10^{15} PFU/ml. Our study provides detailed analysis of isolated *S. suis* lytic bacteriophage and its partial characterization, which could be a natural therapeutic strategy against antimicrobial resistant *S. suis* strains.

Introduction

In the health aspect of porcine, there are serious increases in emergence of porcine bacterial diseases now days. One among is Streptococcal infection mainly caused by *Streptococcus suis* leads to financial losses in the swine industry (Haas and Grenier, 2018) due to morbidity and mortality. It has zoonotic potential associated with septicaemia, arthritis, meningitis and septic shock in both pigs and humans (Higgins and Gottschalk, 2005). Its public health importance was highlighted by a recent

large-scale outbreak of human *S. suis* infections in China in 2005, which resulted in 38 deaths (Yu *et al.,* 2006). *S. suis* is a gram-positive facultative anaerobic coccus, originally defined as Lancefield groups R, S, R/S or T. Later, a new typing system based on the type specific capsular polysaccharide antigens located in the cell wall was proposed comprising 35 serotypes (types 1-34 and ½) of which serotypes 2, 1, 9, 7 and 1/2 are the most prevalent (Rasmussen and Andresen, 1998). Serotype 2 of *S. suis* is considered to be the most virulent and is most frequently isolated from clinically-diseased piglets (Staats *et al.,* 1997).

Serotype 9 is the most prevalent serotype causing invasive disease in pig population of several European countries (Segura *et al.,* 2016) and this serotype is carried by the majority of healthy pigs in Europe (Segura *et al.,* 2017). In many cases, more than one serotype can colonize a single pig as seen in a study by Flores et al (1993). Dawei et al (2012) provided the detailed analysis of *S. suis* biofilm formation potential that could be a target mechanism where the bacteriophages could play a major therapeutic role in inhibiting the biofilms and eradicating the disease.

Therefore, it is necessary to investigate and determine the biological characteristics of phages for practical therapeutic applications and to expand our understanding of phages as alternatives to antibiotics. With the goal of improving treatment and prevention of *S. suis* infection, we isolated, characterized lytic phage against *S. suis* strains

Materials and Methods

All the bacterial strains used in the study (Table 2) were cultured in Brain heart infusion broth (BHI, Himedia) supplemented with 10% foetal bovine serum (Genetix), growth characteristics were observed on 5% sheep blood agar and strains were maintained on blood agar plate at 4^{o}C by subcultured fortnightly. The growth of the organism is enhanced in microaerophillic conditions using CO_2 gas generators.

Phage isolation, Purification and Propagation

To isolate phages infecting *S. suis* serotype 2, 50 sewage samples from piggery farm, ICAR-IVRI, Izatnagar were collected over the span of 2 months, centrifuged at 6000 rpm for 15 min and supernatant was filtered through 0.22µm membrane filters (Axiva). *S. suis* serotype 2 strain was used as an indicator host strain. To isolate phages, 6ml of filtered sewage samples were inoculated with 3ml of overnight grown culture of host strain and 6ml of double strength NZCYM broth (Himedia) and cultured for 24 h at 37 ^{o}C in shaker incubator. After enrichment, the presence of the phage was verified by spotting the diluted cultures on the top agar containing the bacteria.

Cultured samples that showed inhibition zones were picked and crushed the plaques by suspending in Sodium chloride and Magnesium sulphate buffer (SM buffer), were centrifuged at 10,000 ×g for 20 min and the resultant supernatant was filtered through 0.22µm membrane filter. To confirm the presence of lytic phages in the filtrate, double strength layered agar overlay method was performed with some modifications (McDuff *et al.,* 1961). Briefly, the filtrate was added to the 18 h grown culture of host bacteria in the presence of $CaCl_2$ solution, incubate at 37 ^{o}C for 20 min in shaker incubator and add mixture to 0.75% semisolid NZCYM agar, spread evenly on the top NZCYM agar. The plates were examined for the plaques. These plaques were purified 2-3 times through single plaque isolation with sterile microtip to

ensure that the isolated phages were descendents from the single virion. *S. suis* serotype 2 phages formed clear, uniform plaques and the phage propagation was done by pouring 5ml of SM buffer in agar overlay plate showing uniform plaques. Plaques were disturbed with sterile microtip and incubated at 37 ^{o}C for 8 hours. The mixture was centrifuged at 5000rpm for 15 min and filtered through 0.22µm membrane filter. The filtered phage suspension was stored at 4 ^{o}C and then several of such stock preparations were selected for further study and designated as SS1, SS2, SS3 and SS4 phage stock preparations.

Host range analysis

The host range of all the phages were determined using a spot assay and confirmed by the double layer agar method against other serotypes of *S. suis* organisms, various gram positive and gram negative organisms (Table 2). 10µl of the phage lysate was dropped on to the agar plate containing 0.75% NZCYM semisolid agar with each of the bacterial strain. The plates were then dried for 5 min at room temperature and incubated overnight at 37 ^{o}C and checked for the lysis zone.

Phage morphology and plaque forming unit count determination

The plaques were evaluated on the basis of their size and shape. The phages were serially diluted10 fold in SM buffer. Equal quantity of 100µl of each phage dilution and overnight grown culture of *S. suis* serotype 2 and 100mM of sterile $CaCl_2$ solution were vortexed for a minute and allowed to stand at 37 ^{o}C for 20 min in shaker incubator. The contents were mixed in 3ml of 0.75% semisolid NZCYM agar and poured on top NZCYM agar plates. The plates were then incubated at 37 ^{o}C for 24 h for the presence of plaques and count the plaques (PFU/ml).

pH stability assay

The effect of change in pH on survivability of phage was observed by subjecting phage to variable pH conditions of 2, 7 and 11 in SM buffer. 100µl of stock phage preparation was mixed with 900µl of SM buffer having pH 2,7 and 11 respectively and were kept at 4 ^{o}C. After exposure of 30 min to various pH conditions, aliquots of phage preparations were subjected to phage titer (PFU/ml) estimation to determine the survivability of phage.

Thermal stability assay

The effect of temperature on phage was observed by subjecting 200µl of stock phage preparation at various temperatures of 4 ^{o}C,-20 ^{o}C, 20 ^{o}C, 37 ^{o}C and 60 ^{o}C and exposed for 30 min. Then the aliquots of phage preparations at each temperature conditions were subjected to phage titer (PFU/ml) estimation to determine the phage survivability.

Results and Discussion

The phages of *S. suis* 2 were isolated from piggery sewage water samples after enrichment. Phages were designated as SS1, SS2, SS3 and SS4 phages. Three different phage morphotypes were observed against *S. suis* 2 (Fig. 1, 2 & 3). The diameter of the plaques of each phage was measured and SS2 phage was found to have the larger plaque size of 4mm (Table 1). Among all four phages, the SS2 phage was found to have the highest phage titre of 14.3 Log PFU (Table 1). Host range for the phages was determined against different bacterial strains (Table 2). It was observed that SS1, SS2 and SS3 phages were showing lytic activity against serotypes of *S. suis*. However, SS4 phage was only lytic against *S. suis* serotype 2 and 7. SS1, SS2 and SS3 phages were lytic to methicillin resistant

Staphylococcus aureus (MRSA), *Streptococcus pyogenes*, *Streptococcus dysgalactiae* and *Enterococcus* sp. None of the phage shown infectivity against *Escherichia coli*, *Salmonella* Gallinarum and *Yersinia enterocolitica*.

Table.1 Plaque diameter and phage titre of *S. suis* phages

***S. suis* Phages**	**Diameter (mm)**	**Log PFU count**
SS1 (SS2)	3	10
SS2 (SS2)	4	14.3
SS3 (SS2)	3	10.47
SS4 (SS2)	Pinpoint plaques <1	12.30

Table.2 Host range and lytic activity of *S. suis* phages on different bacterial strains

Bacterial Species	**Lytic activities of phages**			
	SS1	**SS2**	**SS3**	**SS4**
***S. suis* Serotype 2**	+	+ +++	+	+
***S. suis* Serotype 1/2**	+	+ ++	+	-
***S. suis* Serotype 3**	+	+ ++	+	-
***S. suis* Serotype 7**	+	+++	+	+
***S. suis* Serotype 9**	+	+++	+	-
Methicillin resistant Staphylococcus aureus	+	+	+	-
Streptococcus dysgalactiae	+	+	+	-
Enterococcus sp.	+	+	+	-
Streptococcus pyogenes	+	+	+	-
Escherichia coli	-	-	-	-
***Salmonella* Gallinarum**	-	-	-	-
Yersinia enterocolitica	-	-	-	-

Fig.1 Plaque morphology of SS2 phage

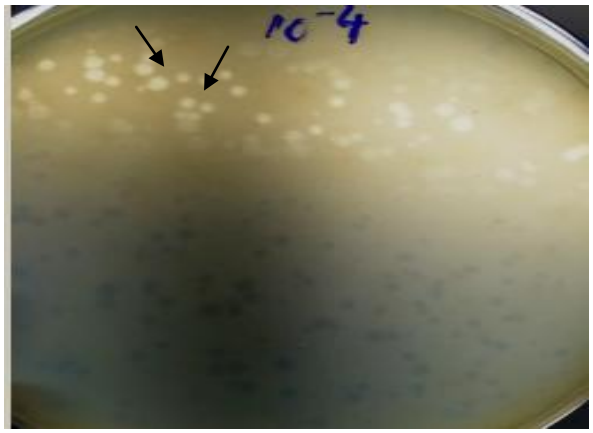


Fig.2 Plaque morphology of SS1 and SS3 phage

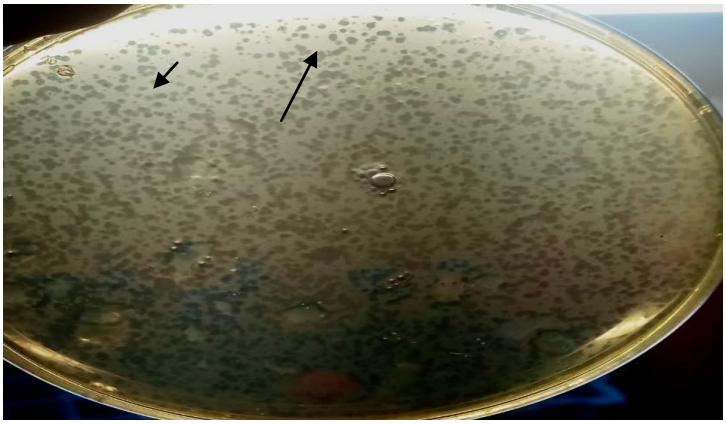


Fig.3 Plaque morphology of SS4 phage

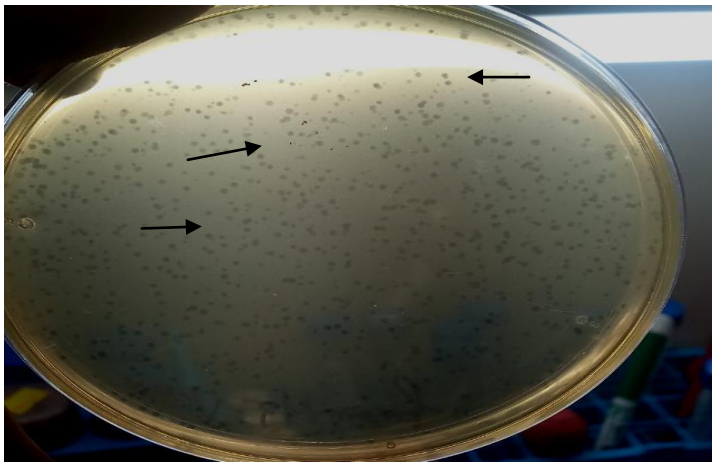


Fig.4 Temperature stability of SS2 phage on incubation for 30 min at different temperature

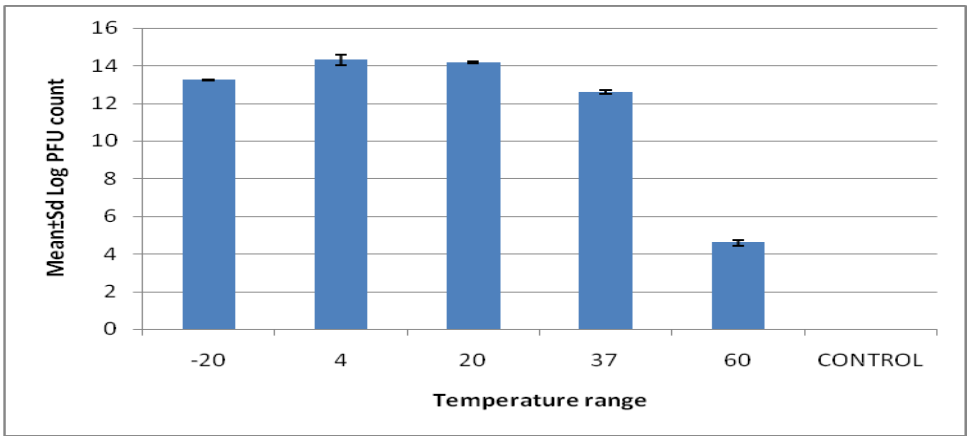
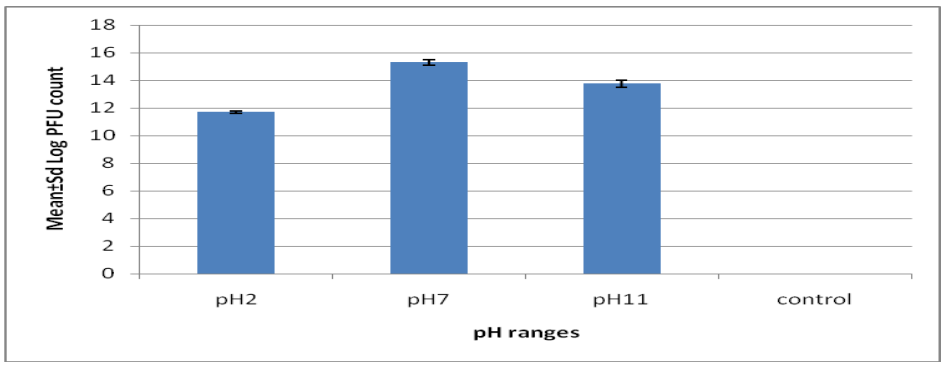


Fig.5 pH stability of SS2 phage on incubation for 30 min at different pH



In the present study, out of the 4 isolated phages only SS2 phage was characterized further. The short term thermal stability tests showed that SS2 phage was stable at 4 ^{o}C, 20 ^{o}C,-20 ^{o}C and 37 ^{o}C for 30 min with highest lytic activity at 4^{o}C with phage titer 2×10^{14}PFU/ml, but the phage numbers were significantly dropped at 60^{o}C with phage titer 4×10^{4}PFU/ml (Fig. 4). Upon pH stability tests, SS2 phage was showing stability at pH 2, 7 and 11 for 30 min with highest lytic activity at pH 7 with counts of 2×10^{15}PFU/ml (Fig. 5). Additionally, on chemical stability assay, SS2 phage showed strong lytic activity with complete clearance of bacteria at 10^{-10} dilution on overnight incubation.

Among emerging porcine bacterial zoonoses the streptococcal infections caused by *Streptococcus suis* in pigs are trending recently (Haas and Grenier, 2018). *S. suis* is the major pathogen of piglets ageing 5-10 weeks old with clinical manifestation of septicaemia, meningitis, endocarditis and death (Segura *et al.*, 2016) and its serotype 2 is considered to be the most virulent and most frequently isolated from piglets worldwide (Staats *et al.*, 1997).

Consequently, development in multidrug resistant bacteria and decrease in the efficacy of antimicrobial therapy led to focus on newer therapeutic strategies. One such study is phage based therapies which focus mainly on lytic phages that destroy their bacterial hosts in a specific manner (Tinoco *et al.*, 2016). The only lytic *S. suis* phage SMP was reported so far (Wang *et al.*, 2009).

In the present study, the isolated lytic *S. suis* phages infects multiple serotypes of *S. suis* organism and the phage (SS2 phage) that produced larger plaque size, was further characterized for its biological properties by subjecting the phage to various temperatures, pH and chemical conditions. Moreover, SS2 phage was found to be stable at pH 2,7 and 11 with highest lytic activity at pH 7 and also found to be stable at4 ^{o}C,-20 ^{o}C, 20 ^{o}C and 37 ^{o}C for 30 min with highest lytic activity at 4 ^{o}C. One of the most critical aspects in using phage for therapeutics is to reduce the recurrence of bacterial growth by allowing to amplify the phage in the host system as a natural biocontrol agent. Indeed, the isolated SS2 phage was able to inhibit host bacteria completely with clear lysis on spot assays throughout the study. It confirms that the effectiveness of SS2 phage and bacterial susceptibility to phage was maintained. This study supports the possibility of implementing SS2 phage and their lysins as biocontrol agents. Phages are unique in their greater specificity to the host organism with specific receptor. In fact, in the present study SS2 phage was able to lyse other streptococcal organisms such as *Streptococcus pyogenes* and *Streptococcus dysgalactiae* and also able to lyse Methicillin resistant *Staphylococcus aureus* and *Enterococcus* sp. Similar findings of phages infecting bacteria from different genera were reported by Pantucek *et al.*, (1998) and Ross *et al.*, (2016).

In conclusion, the present study characterized *S. suis* serotype 2 phage. This helps in implementing bacteriophages in application of biocontrol. Our findings support the potential of SS2 phage in controlling swine and human streptococcal infections caused by *S. suis*. Further characterization of phages and isolating various type of phages could help in implementing cocktail therapy to expand antibiotic therapy is imperative.

Acknowledgements

We give sincere appreciation and thankful for the work support from Indian Veterinary Research Institute, Izatnagar, Bareilly.

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How to cite this article:

Lahari Laddika, Shumaila Malik, Renu Chauhan, Salauddin Qureshi, Monalisa Sahoo, Sagar Patel and Tiwari, A. K. 2021. Isolation and Partial Characterisation of *Streptococcus suis* Bacteriophage. *Int.J.Curr.Microbiol.App.Sci.* 10(03): 1300-1306.
doi: https://doi.org/10.20546/ijcmas.2021.1003.158