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Isolation and characterization of *Salmonella* bacteriophages from poulty and pig sewage wastes*

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ABSTRACT

Bio-control strategies including bacteriophages as therapeutic agents seem to be cost effective approach to control pathogenic zoonotic infections like *Salmonellosis* in food animals. *Salmonella* specific bacteriophages were isolated, purified and characterized from different locations of sewage samples of swine and poultry. In the present study, two sewage lagoons i.e. one poultry farm sewage and one sewage waste yielded plaques of different sizes. Some of the plaques were clear while others were turbid. From these different plaques, 5 different bacteriophages were isolated and their genome was estimated to about 42 kbp in length. The bacteriophages were characterized with the help of restriction enzyme analysis and transmission electron microscopy. Bacteriophages named PSP 4, PSP 5 and PSP 7 belonged to family Siphoviridae and PSP 1 and PSP 6 were a member of family Podoviridae. Bacteriophages were found to be sensitive to high temperature and low pH. All 5 bacteriophages were subjected to host range analysis. Bacteriophages PSP 7 and PSP 4 had wide host range whereas bacteriophages PSP 1 and PSP 6 were selective in lyses of the bacterial strains. Overall, we have isolated *Salmonella* specific bacteriophages from the poultry and pig environment and this knowledge could be used in bio-control of *Salmonella* pathogen in poultry and pigs.

Key words: Bacteriophages, India, Pig, Poultry, *Salmonella*

Controlling salmonellosis in poultry and pig continues to be problematic and relied historically on a combination of farm biosecurity and use of antibiotics (Davies *et al.* 2005). Concerns about development of antimicrobial resistance and transfer of antibiotic resistance genes from animal to human microbiota have led to withdraw approval for antibiotics as growth promoters in the European Union since 1 January 2006 (Castanon 2007). The ban on antibiotics in animal feeds could severely affect international trade of meat and egg especially from the developing countries like India. Therefore, increasing antibiotic resistance and antibiotic ban in animal food necessities an acceptable and cost effective bio-control strategy to control salmonellosis.

The bacteriophage therapy and competitive enhancement methods like use of probiotics seem to be possible solutions. The bacteriophages are natural predators of bacteria and are ubiquitous in the environment commonly found in water, sewage and soil (Rohwer *et al.* 2002). Bacteriophages infecting *Salmonella* spp. from sewage had been isolated and their potential to control food-borne *Salmonella* had been assessed using soft agar overlays containing three *Salmonella* serovars (Carey-Smith *et al.* 2006). Similarly, Bacteriophages associated with *Salmonella* from 9 swine manure lagoons were isolated in Mississippi, USA. Electron microscopy (EM) showed these purified enrichment isolates had Podoviridae morphology (tailless 50– nm icosahedral heads with tail spikes) (McLaughlin *et al.* 2006). During year 2004–2005, 232 phages were recovered from poultry farms, abattoirs, and wastewater and 3 of isolated phages are broad range phages targeting *Salmonella* serovars such as Enteritidis, Hadar and Typhimurium (Atterbury *et al.* 2007).

The present study describes collection of poultry and pig sewage puddles for isolation and characterization of *Salmonella* bacteriophages. To our knowledge, this is the first report of isolation and characterization of various bacteriophages available in the local sewage from Uttarakhand, India.

MATERIALS AND METHODS

Collection of effluent from sewage puddles: Samples were collected from chicken excretion sewage and sewage puddles, where both poultry and swine waste accumulates from 5 different locations in and around Pantnagar, Uttarakhand (India). About 20 mL sample was drawn every time from a depth of about 15 cm using a long sterile glass

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pipette fitted with pipette bulb. *Salmonella* Typhimurium (TG7) previously isolated from poultry was used for bacteriophage enrichment.

Bacteriophage isolation, purification and amplification: Agar overlay technique was used to isolate bacteriophage as described by Adams (1959). Bacteriophages were purified by successive single plaque isolation. Bacteriophage sensitivity was tested by double layer plaque assay and spot-on test (Kutter *et al.* 2005). All bacteriophages used in present investigation were amplified as per Oliveira *et al.* (2009). The resultant bacteriophage suspension was filtered through 0.22 µm syringe filter and stored at 4°C.

Temperature and pH stability of bacteriophages: To test their heat stability, 1.5 ml tubes full with bacteriophages (10^{11} PFU/ml) were kept in a water bath at 30° to 70°C temperature for 15, 30 and 60 min. Bacteriophages mixed with TSB of different pH (3 to 11, adjusted with NaOH or HCl) were incubated for 1 h at 37°C, to ascertain their pH stability. Bacteriophage titers were determined using double layer agar plate method.

Transmission electron microscopy (TEM) of bacteriophages: Morphology of isolated bacteriophages was studied using electron microscopy (Microscope électronique à transmission JEOL, modèle JEM1010). Negative staining was done as per Turki *et al.* (2012).

Bacteriophage nucleic acid isolation and characterization: Salmonella bacteriophage nucleic acid was extracted as per Sambrook *et al.* (2002) with minor modification. Purified DNA was re-suspended and stored at –20°C for further use. Different bacteriophages were characterized by digesting the extracted and purified DNA with restriction enzymes *Eco*RI and *Hind*III, individually. Gel was visualized under UV light and the restriction profile and fragment sizes of isolated bacteriophages DNA was documented by gel documentation system (AlphaImager^{R} HP-2200 Documentation and Analysis System, Alpha Innotech Corporation, USA).

Host strain range determination of bacteriophages: The host range of each bacteriophage isolate was determined by spot-on test using different *Salmonella* strains. From a total of 58 *Salmonella* strains used, 51 *Salmonella* strains isolated from field include Typhimurium TGP (Poultry) (n = 20), Typhimurium TGS (Swine) (n = 5), Enteritidis TGP (n = 16), Gallinarum TGP (n = 7), Infantis TGS (N = 1), and Bredney TGS (n = 2) while 7 *Salmonella* strains (*Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Gallinarum, *Salmonella* Pullorum, *Salmonella* Cholerasuis, *Salmonella* Infantis and *Salmonella* Bredney) were obtained from IVRI Izatnagar, India.

RESULTS AND DISCUSSION

Bacteriophages isolated from sewage samples: Out of the 5 different locations of sewage puddles, only 2 (1poultry farm sewage and 1 sewage where both swine and poultry waste accumulates) yielded plaques (Table 1). Plaque formation was evident after 4–5 h of incubation but differences in plaque morphology, and sizes were evident only after 12 h of incubation. Number of plaques increased after 24 h of incubation i.e. double the number present after 12 h. Present investigation revealed heterogeneous mixture of plaques having large, medium and small diameter. Also, some plaques had clear zones and others were turbid. Five different plaques based on the size and clarity were selected for bacteriophage isolation. From different plaques, 5 different bacteriophages were isolated and purified. Name of the bacteriophage isolates included phage, host strain, place of research and identification number. For example, in naming PSP1, the letter P stands for phage, the letter S is for *Salmonella,* P stands for Pantnagar (place) and the number 1 represents the initial identification number. Five different bacteriophages selected for the further experiments were PSP1, PSP4, PSP5, PSP6 and PSP7.

Heterogeneous mixture of plaques having large, medium and small sizes was evident in the first passage suggested that different types of bacteriophages were present in raw sewage sample from 2 separate sites. According to Stent (1963), the diameter of the plaque is influenced by bacterial host, the nature of the bacteriophage, plating and incubation conditions. Clear plaques were indicative of virulent bacteriophages and broad host range while lysogenic bacteriophages apparently could be identified by turbid plaques and have a narrow host range (Adams 1959). Several other factors like the physiological state of the host and temperature and pH of media determine the plaque morphology in terms of the clarity of the plaques (Kutter *et al.* 2005).

Table 1. Information on phage isolates from sewage samples

Name of phage isolate	Original host bacteria	Plaque size (mm)
PSP1*	*Salmonella* Typhimurium	1.0
PSP4*	*Salmonella* Typhimurium	4.0
PSP5*	*Salmonella* Typhimurium	5.0
PSP6*	*Salmonella* Typhimurium	4.0
PSP7**	*Salmonella* Typhimurium	2.0

Source of phage (name of place), * swine and poultry effluent (Pantnagar); **, Poultry effluent (Bajpur).

Heat and pH stability of isolated bacteriophages: For heat and pH stability studies, the titers of bacteriophages namely PSP1, PSP4, PSP5, PSP6 and PSP7 were all adjusted to 10^{11} PFU/mL. The titers of bacteriophages were stable when heated at different temperatures ranging from 30°C to 60°C for 15, 30 and 60 min (Table 2). Maximum stability was observed at the temperature ranging from 30 to 50°C. As the temperature increased beyond 60°C bacteriophages activity start decreasing and at 70°C no bacteriophages activity was observed.

The activity of 5 bacteriophages was relatively stable at pH 6 to 9 and declined dramatically at high as well as low

Table 2. Effect of temperature on the stability of bacteriophages

Temperature (°C)	Bacteriophages with concentration (Log 10)														
	PSP1			PSP4			PSP5			PSP6			PSP7		
	15 min	30 min	60 min	15 min	30 min	60 min	15 min	30 min	60 min	15 min	30 min	60 min	15 min	30 min	60 min
30	9.5	9.0	8.5	9.5	9.4	9.4	9.5	9.4	9.4	9.5	9.4	9.4	9.5	9.4	9.4
40	10.8	10.7	10.4	10.9	10.8	10.6	10.9	10.8	10.6	10.9	10.8	10.6	10.9	10.8	10.6
50	10.7	10.5	10.4	10.9	10.8	10.6	10.9	10.8	10.6	10.9	10.8	10.6	10.9	10.8	10.6
60	9.0	8.8	8.3	9.0	8.8	8.3	9.0	8.8	8.3	9.0	8.8	8.3	9.0	8.8	8.3
70	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

pH. No bacteriophage activity was detected at pH 3 and 11. Five bacteriophages were isolated and further characterized. In term of heat, maximum stability was observed at the temperature ranging from 30° to 50°C. Temperature beyond 60°C saw the loss in bacteriophage activity, and at 70°C no activity was observed. Similar findings were observed earlier (Chandra *et al.* 2011)**.** Effect of pH in the stability of bacteriophages is a major finding as bacteriophages have to pass through proventriculus and gizzard having low pH, when given orally. In our study, it was revealed that the isolated bacteriophages were sensitive to low pH. Similar findings had also been previously recorded (Carvalho *et al.* 2010, Kwiatek *et al.* 2012).

Bacteriophage nucleic acid isolation and characterization: The restriction enzymes digestion of bacteriophage DNA allows the approximate determination of the genomic size of bacteriophages (Bao *et al.* 2011).The genomic nucleic acid of all the 5 bacteriophages was isolated and a bright, clear band was observed above the band size of 23 Kb of molecular size marker. Overall 12 different bands ranging from the size 23,000 to 2,000 bp were observed when purified genomic DNA of all 5 bacteriophages were digested with *Eco*RI. Two strong bands between 23,000 and 9,400 were observed with *Eco*RI. However digestion pattern was not very clear with *Eco*RI as many indiscriminate overlapped bands were present (Fig. 1). The enzyme *Hind*III generated more discriminatory bands with all the bacteriophages. Seven distinct bands ranging from the size 23,000 and 500 bp were observed. Three strong bands between 23000 and 4500 were observed with *Hind*III. Also, 4 bands of smaller size ranging between 4000 and 500 were clearly visible. On the basis of results of restriction fragment analysis (Fig. 2), bacteriophages genome was estimated to be about 42 kbp for all 5 isolated bacteriophages.

Isolated bacteriophages were further characterized by restriction fragment analysis and TEM. Restriction digestion of isolated bacteriophages with *Hind*III followed by agarose gel electrophoresis estimated that the size of bacteriophages genome to be about 42 kbp for all 5 isolated bacteriophages. TEM characterized bacteriophages into tail-less (PSP 1 and PSP 6) and tailed ones (PSP 4, PSP 5 and PSP 7). Similarly, Lappe *et al*. (2009) characterized 16 bacteriophages belonging to Podoviridae, Siphoviridae and Myoviridae by

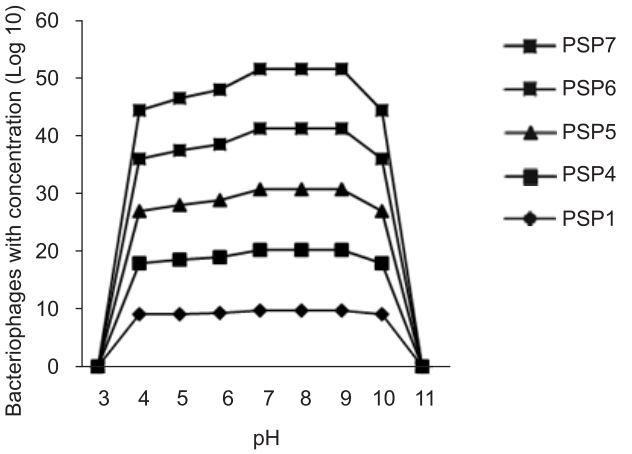

Fig. 1. Effect of pH on stability of bacteriophages.

gel electrophoresis following restriction endonuclease digestion of the genome with *Hind*III. The genome sizes for the Podoviridae and Siphoviridae (PFGE-A) were approximately 42 kb.

Transmission electron microscopy (TEM) of bacteriophages: TEM images of 5 *Salmonella* bacteriophages revealed tailed and non-tailed phages (Fig. 3a,b).

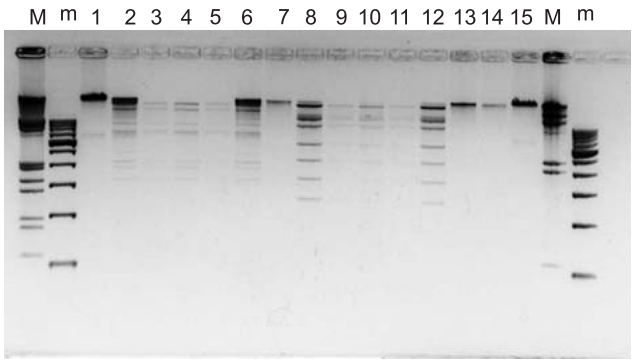

Fig. 2. Restriction enzyme analysis of Bacteriophages DNA with *EcoRI* and *HindIII*. Lane M = 23-kb DNA molecular size marker; Lane m= 500 bp DNA molecular size marker. Lane 2-6 = Digested DNA of PSP1, PSP4, PSP5, PSP6 and PSP7 with *EcoRI* ; Lane 8-12 = Digested DNA of PSP1, PSP4, PSP5, PSP6 and PSP7 with *HindIII*. Lane 1, 7, 13, 14 & 15= Undigested DNA of Bacteriophages PSP1, PSP4, PSP5, PSP6 and PSP7 respectively

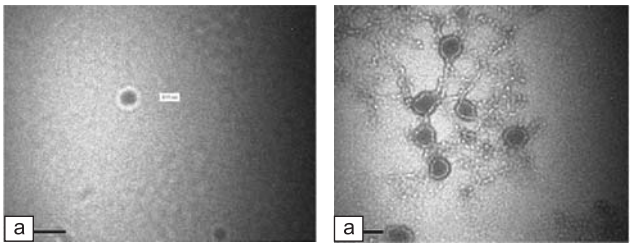

Fig. 3a Transmission electron microscopy of bacteriophage PSP1. **3b** Transmission electron microscopy of bacteriophage PSP5.

Both PSP 1 and PSP 6 were without tail, whereas PSP 4, PSP 5 and PSP 7 had tail. The tail-less PSPs, PSP1 and PSP6, were characterized as follows. The capsid diameter of bacteriophage PSP 1 was measured approximately 103 nm between opposite apices and was icosahedral in shape. The capsid diameter of bacteriophage PSP 6 was measured approximately 63 nm between opposite apices and was elongated in shape. The tailed PSPs, PSP 4, PSP 5 and PSP 7, had the following characteristics. The capsid of bacteriophage PSP 4 was isometric in shape with diameter measured approximately 110 nm between opposite apices and tail length of about 55 nm. The capsid of bacteriophage PSP 5 was icosahedral in shape with diameter measured approximately 70 nm between opposite apices and tail length of about 90 nm. The capsid of the bacteriophage PSP 7 possessed isometric shape with diameter measured approximately 50 nm between opposite apices and tail was thin, flexible with length of about 122 nm. The differences in capsid size of some bacteriophages suggest that bacteriophage isolates belonged to different morphoypes within the same family. Based on morphological analysis by TEM (Fig 3a,b), all 5 bacteriophages belonged to the order Caudovirales. In the order Caudovirales, bacteriophages PSP 4, PSP 5 and PSP 7 to the family Siphoviridae while bacteriophages PSP 1 and PSP 6 were categorized under the family Podoviridae.

Our TEM results showed agreement with the fact that sewage samples were rich in bacteria and the viruses that infect them. A number of bacteriophage types were found, most belonging to the families Siphoviridae and Podoviridae (Ackermann 2005). The present study showed that the bacteriophages PSP 1 and PSP 6 isolated from effluent from Pantnagar (India) belong to the Podoviridae family. McLaughlin et al. (2006) showed that purified enrichment bacteriophage isolates collected from swine effluent lagoons had Podoviridae morphology. Tail-less phages observed in present study probably had short tail that was not visible. However, this is only speculative and cannot be concluded from the electron micrograph. It has also been reported that osmotic shock could destroy viral particles such as tail fibers and other structures (Konopa et al. 1979). The morphology observed in such cases might offer incomplete information and could affect identification purposes. Bradley (1967) emphasised that no morphological bacteriophage type is unique to any particular bacterial genus or species and

Table 3. Host range of 5 *Salmonella* bacteriophage isolates determined on 25 *Salmonella* Typhimurium host strains

Salmonella Typhimurium strain	Bacteriophage isolates				
	PSP1	PSP4	PSP5	PSP6	PSP7
TG 4	+	+	+	+	+
TG 5	±	+	+	–	+
TG 6	+	+	+	+	+
TG 7[a]	–	±	–	–	+
TG 39	±	+	+	–	+
TG 40	+	+	+	+	+
TG 41	–	–	±	+	±
TG 43	+	+	+	+	+
TG 44	±	+	+	–	+
TG 46	+	+	+	+	+
TG 47	–	±	–	–	+
TG 50	±	+	+	–	+
TG 51	+	+	+	+	+
TG 52	–	–	±	+	±
TG 53	+	+	+	+	+
TG 55 [a]	±	+	+	–	+
TG 56 [a]	+	+	+	+	+
TG 60	–	±	–	–	+
TG 62 [a]	±	+	+	–	+
TG 66	+	+	+	+	+
TG 67 [a]	–	–	±	+	±
TG 68	+	+	+	+	+
TG 72	±	+	+	–	+
TG 74	+	+	+	+	+
TG 75	–	±	–	–	+

TG, identification identity of *Salmonella* isolates given by author. [a]Strains isolated from pigs. (+) clear spot on diameter of the inoculated area; (±) faint spot within the inoculated area; (–) no spot formation.

Table 4. Host range of 5 *Salmonella* bacteriophage isolates determined on 16 *Salmonella* Enteritidis host strains

Salmonella Enteritidis strain	Bacteriophage isolates				
	PSP1	PSP4	PSP5	PSP6	PSP7
TG 25	+	+	+	+	+
TG 27	±	+	+	–	+
TG 37	+	+	+	+	+
TG 38	–	±	–	–	+
TG 42	±	+	+	–	+
TG 45	+	+	+	+	+
TG 48	–	–	±	+	±
TG 49	+	+	+	+	+
TG 54	±	+	+	–	+
TG 57	+	+	+	+	+
TG 58	–	±	–	–	+
TG 63	±	+	+	–	+
TG 64	+	+	+	+	+
TG 69	–	–	±	+	±
TG 73	+	+	+	+	+
TG 76	±	+	+	–	+

TG, identification identity of *Salmonella* isolates given by author. (+) clear spot the diameter of the inoculated area; (±) faint spot within the inoculated area; (–) no spot formation.

Table 5. Host range of 5 *Salmonella* bacteriophage isolates determined on 7 *Salmonella* Gallinarum host strains

Salmonella Gallinarum strain	Bacteriophage isolates				
	PSP1	PSP4	PSP5	PSP6	PSP7
TG 1	+	+	+	+	+
TG 2	±	+	+	–	+
TG 3	+	+	+	+	+
TG 19	–	±	–	–	+
TG 70	±	+	+	–	+
TG 71	+	+	+	+	+
TG 77	–	–	±	+	±

TG, identification identity of *Salmonella* isolates given by author. (+) clear spot in the diameter of the inoculated area; (±) faint spot within the inoculated area; (–) no spot formation.

Table 6. Host range of 5 *Salmonella* bacteriophage isolates determined on 2 *Salmonella* Bradeney and 1 *Salmonella* Infantis host strains isolated from pigs

Salmonella strain	Bacteriophage isolates				
	PSP1	PSP4	PSP5	PSP6	PSP7
Salmonella Bradeney (TG 59)	–	+	±	–	+
Salmonella Bradeney (TG 65)	–	+	±	–	+
Salmonella Infantis (TG 61)	–	+	±	–	+

(+) clear spot in the diameter of the inoculated area; (±) faint spot within the inoculated area; (–) no spot formation.

Table 7. Host range of 5 *Salmonella* bacteriophage isolates determined on 7 *Salmonella* serotypes purchased from Indian Veterinary Research Institute

Salmonella Serotype	Bacteriophage isolates				
	PSP1	PSP4	PSP5	PSP6	PSP7
Salmonella Gallinarum	+	+	+	+	+
Salmonella Pullorum	–	+	+	+	+
Salmonella Enteritidis	–	+	+	+	±
Salmonella Typhimurium	±	+	+	+	+
Salmonella Infantis	–	+	–	–	+
Salmonella Bredeney	–	+	–	–	+
Salmonella Cholerasuis	±	+	+	+	±

(+) clear spot in the diameter of the inoculated area; (±) faint spot within the inoculated area; (–) no spot formation.

therefore one might find bacteriophages, which are similar morphologically but infect different bacteria. Price and Rooyen (2001) noted that the presence of debris in the sample can easily mask certain structures on the bacteriophages and useful information regarding the isolated bacteriophages could be lost. Similar hurdles were experienced in our study for some of the images. So negative staining and TEM could be a challenging technique and the degree of success with results could be inconsistent depending on the phage titre, amount of stain used and timing involved in staining the sample during preparation. Despite all the challenges, it remained a useful technique for presumptive phage classification (Ackermann et al. 1992). On the basis of both phage DNA analysis and TEM images we can presumptively identify them as belonging to families Siphoviridae and Podoviridae.

Host range of bacteriophages: The 5 characterized bacteriophages were used to determine the host range spectrum (Tables 3–7). Spot-on test revealed that most of bacteriophages showed lytic activity against all the *Salmonella* strains used in the study. Host range analysis revealed that bacteriophages PSP 4, PSP 5 and PSP 7 isolated from different sources were able to lyse maximum number of *Salmonella* strains whereas bacteriophages PSP 1 and PSP 6 were selective in lysing bacterial strains.

In our study, all the 5 bacteriophages especially PSP 7 and PSP 4 showed wide host ranges. Kocharunchitt *et al.* (2009) showed that bacteriophages SSP5 and SSP6 have broad host ranges of over 65% of the 41 *Salmonella* strains tested. Isolation of 5 bacteriophages from poultry faeces and concluded that 2 of the isolated bacteriophages lysed approximately 44.6% and 48.0% of avian pathogenic *E. coli* strains had previously reported as well (Oliveira *et al.* 2009). The narrow host range had been shown by phage isolates PSP6 and PSP1. This state of certain bacteriophages might be because of resistance adopted by bacterial strains which may be because of lack of suitable receptors or binding sites or as a result of mutations in bacteriophage or bacteria (Adams 1959). The presence or absence of receptors on the bacterial membrane too plays a crucial role in determining whether bacterial lysis will take place or not (Engelkirk and Burton 2002). If the bacteria have many recognised receptors greater number of plaques will be observed on the agar plates. It could be the possible reason why different lytic patterns were observed between the different bacterial strains. Host range analysis and transmission electron microscopy revealed differences among bacteriophages. Those 5 bacteriophage strains, isolated from 2 different sources could be closely related but diverged enough to change host range. Bigwood *et al.* (2008) found that both host range and adsorption rate are genetically determined and that changes in host range are common.

We have successfully isolated lytic bacteriophages from 2 different locations out of total 5 different sewage puddles. The presence of bacteriophages was maximum in puddles where mixture of swine and poultry waste accumulates. All the 5 isolated bacteriophages showed wide host range especially PSP 7 and PSP 4.The bacteriophages genome was estimated to about 42 kbp in length for all the 5 isolated bacteriophages.Transmission electron microscopy was found to be an extremely useful tool in the morphological characterisation of bacteriophages.

Salmonella specific bacteriophages were isolated,

purified and characterized to control pathogenic zoonotic infections like salmonellosis in food animals. In the present study, 2 sewage lagoons were used to isolate plaques and from these different plaques, 5 different bacteriophages were isolated. These bacteriophages were characterized with the help of restriction enzyme analysis and transmission electron microscopy and found belonging to families Siphoviridae and Podoviridae. Isolated bacteriophages exhibited narrow to wide host range in term of lysis of bacterial strains. Overall, we have isolated and characterized *Salmonella* specific bacteriophages from sewage lagoons and this knowledge could be used in bio-control of *Salmonella* pathogen in poultry and pigs.

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