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Sanem Soma Sekhar Goud
Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, PV Narasimha Rao Telangana Veterinary University, Hyderabad, Telangana, India

A Jagadeesh Babu
Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

V Sasikala
Final year B.V.Sc. & A.H. students, College of Veterinary Science, Tirupati, Andhra Pradesh, India

R Surendra
Final year B.V.Sc. & A.H. students, College of Veterinary Science, Tirupati, Andhra Pradesh, India

Correspondence
A Jagadeesh Babu
Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

Studies on the lytic bacteriophages against *Escherichia coli* isolated from dairy farm sewage

Sanem Soma Sekhar Goud, A Jagadeesh Babu, V Sasikala and R Surendra

Abstract
The objective of this study was to isolate and characterize bacteriophages against *Escherichia coli* isolated from dairy farm sewage. The sewage samples (N=128) from organized, unorganized dairy farms and also from animal sheds with two to five number of cattle's were collected and cultured to isolate *Escherichia coli*. The sewage samples were centrifuged and filtered using 0.22 µm filters and the lytic phages were enriched and purified against *E. coli* and characterized. Survival rate of target bacteria was assessed by inoculating phage in the actively growing log phage host. *E. coli*, which is native of sewage samples, was isolated and subjected to biochemical tests. A total of 22 *E. coli* phages were isolated. The SEM observations of phage revealed that the phage had icosahedral head with short tail. The in-vitro experiment showed that there was a 100% reduction in the *E. coli* count after 10 hours of incubation. Bacteriophages are obligate intracellular parasites that multiply inside bacteria. Bacteriophages have been used in many applications including phage therapy and in control of food borne pathogens. The bacteriophages are used as an alternative bio-control agent because they are active against bacteria that have become resistant to antibiotics.

Keywords: lytic bacteriophages, Escherichia coli, dairy farm sewage

Introduction
Enteric bacteria like *Escherichia coli, Shigellasps*, enterotoxigenic *E. coli* (ETEC), etc. Petri *et al*., (2008) [30] infect gastrointestinal tract of humans and animals. Sewage from waste water streams and animal farms are the major sources for enteric pathogens Beaudoin *et al*., (2007) [7]. The treated waste water used as water resources in agricultural and industrial applications, is an origin for re-emergence and distribution of pathogenic bacteria Baggi *et al.*, (2001) [5]. Coliforms are responsible for infectious diseases in humans and animals frequently among sewage pathogens Baggi *et al*., (2001) [5]. Amongst coliforms *E. coli* is the contamination index of water, food and agricultural products, indicating contamination with coliforms and waste water Reid *et al*., (2001) [31]. The World Health Organization (WHO) estimated that five million children die each ear as a consequence of acute diarrhoea Snyder and Merson, (1982) [40]. *E. coli* has one of the widest spectra of disease of any bacterial species, is a evidence of its malleable genetic character Donnenberg (2002) [16].
Bacteriophages are viruses, ranges from 24-200 nm in size Al-Mola and Al-Yassari (2010) [3]. These are an obligate intracellular parasite, which requires specific bacteria for replication Carlton (1999) [12]. Similar to other viruses, bacteriophages have a protein coat, capsid surrounding the nucleic acid (RNA or DNA) Grabow (2001) [18].
The host-specificity of phage determined by the receptor sites present on the surface of bacteria. The phage particle enters in contact with its host randomly through passive diffusion and uses the host cell membrane proteins as sites for attachment and entry Campbell (2003) [11]. Following adsorption, the cell wall is rendered penetrable by specialized phage enzymes expressed in the tail or in the capsid. Thereafter, the phage or at least its genetic material, enters the cell an initiates a reproduction cycle. The genetic material can either be integrated into the host genome or remain in the cytoplasm Weinbauer (2004) [45]. During the following stage gene expression, genome replication and morphogenesis occur. New virions progenies are produced and lysed the bacterial cell, typically results from the dual action of peptidoglycan hydrolases (endoysin) and holins, but sometimes the release is achieved by budding or extrusion Weinbauer (2004) [45]. The clinical use of phage therapy is faced with long product development and approval timelines in Western regulatory frameworks.
Keeping in view of the public health significance of *Escherichia coli* the work has been designed to study the isolation and identification of *Escherichia coli* and their specific phages

present in the same environment to develop new strategy for generalized control of bacterial populations, especially pathogens in waste water that come out from animal farms.

Methods

Collection of Samples

The sewage and wastewater samples were collected from different organized and unorganized dairy farms and animal sheds in and around Tirupati. A total of 128 samples from different sources *viz*: Organized dairy farms (n=28), unorganized dairy farms (n=32) and animal sheds (n=68) were collected aseptically in sterilized plastic containers. The collected specimens were processed within 2 to 24 hours of collection.

Isolation and Identification of *E. coli*

Tryptone soy broth (TSB) was used for enrichment of inoculum and incubated at 37^0C. After overnight incubation, the cultures were streaked on MacConkey agar and Eosine Methylene Blue (EMB) agar plates and the plates were incubated at 37^0C for 24h. After incubation the plates were observed for lactose fermenting colonies and greenish metallic sheen colonies respectively. The colonies thus obtained were transferred to nutrient agar slants in duplicate and incubated at 37^0C for 24 h and stored at 4^0C for further identification. For confirmation of *Escherichia coli*, the biochemical tests conducted were triple sugar iron agar test, urease test, motility test and IMViC tests. A loop full of the isolated organism was added to the sterile Luria-Bertani glycerol broth vials and mixed well in vortex mixer. The vials were then labelled and stored until further use.

Bacteriophage isolation

The lytic bacteriophages were isolated by using the disease causing strain *Escherichia coli* as a representative strains employing double agar overlay method described by Adams (1959) [1] with slight modifications using sewage water collected from different dairy farms. As a part of the enrichment process, a portion of sewage sample was mixed with the host bacteria (in log phage) and allowed for incubation overnight at the temperature of 37^0C. The homogenate was centrifuged at 2000 rpm (Remi motor ltd., R8) and followed by filtration using sterile syringe filters (Millex ®GV) with pore size of 0.22µm to make them bacteria-free. After filtration 100 µl of the filtered sample was serially diluted in Salt of Magnesium (SM) buffer, by 10 fold dilution. This filtrate was screened for the presence of Phage and was stored at 4^0C for the further use.

The lysate was then assayed according to the double-agar overlay method of Adams (1959) [1] with slight modifications. The logarithmic phage cells (100µl) of the host bacterial strains in TSB were mixed with 100µl of the serially diluted lysate and were incubated at 37^0C for 20 min. After incubation, 5 ml of 0.75% sterile soft agar was added to this, mixed well and was immediately overlaid on 20% bottom TSA plates. Phage free cultures (containing only bacterial host) and host-free cultures (containing only phage) were used as controls. Then the petri plates were kept in laminar air flow until it gets solidified, and incubator at 37^0C for overnight to obtain clear zone of plaques.

Preparation of bacteriophage stocks

Bacteriophage stocks were prepared by using double agar overlay method with minor modifications (Sambrook *et al.*, 1989) [33]. To the double agar over lay plates that were showing characteristic plaque morphology 3ml of SM buffer was added. The entire surface of semisolid material was scrapped with the sterile spatula and incubated at 37ºC for 8 hours. Then the entire scrapped material with SM buffer was collected with wide bore microtips into 50 ml sterile container. The phage suspension was centrifuged at 10,000 rpm for 15 min at 4ºC and the supernatant was filtered through 0.45µ syringe filter and stored at 4^0C for further use.

Estimation of titre of the isolated bacteriophage against *E. coli*

Titration of the phage was done by preparing 10-fold serial dilution of the phage lysate. For titration, 10^{-1} to 10^{-4} dilutions were made in SM buffer (pH 7.0). Equal quantity of each phage dilution and fresh exponential *Escherichia coli* in SM buffer and subjected to double agar overlay method as described above. Phage titre was determined in terms of plaque forming units (PFU/ml) with the help of formula given below:

$$\text{PFU/ml} = \text{No. of plaques counted} \times \frac{1}{\text{Dilution factor}} \times \frac{1}{\text{volume of phage taken}}$$

Activity of bacteriophages against target bacteria

Survival rate of target bacteria was assessed by inoculating phage in the actively growing log phase host. Sewage sample was collected and filtered. After filtration sewage sample was taken in pre-sterilized flask. Then added the specific phage at 10^6 PFU/ml and used for treatment. The phage inoculated sample was incubated at 37^0C. After periodic time intervals, the phage treated sample was subjected to assess the activity of target bacteria. Sewage sample was taken as control. The following are the treatment sets prepared for the study.

T1 - Sewage water with bacterial isolate
T2 - T1 and host specific bacteriophages

Physico-chemical characterization of isolated bacteriophages

The effect of heat, sunlight and UV light on survivability of phage was determined at different time intervals. Effect of some commonly available chemicals, *viz.*, Sodium do Decyl Sulphate (0.1% and 1%), phenol (1% and 5% aqueous), chloroform (5% and 10%) and formalin (40%) on survivability of bacteriophage was observed.

Phage DNA isolation

The extraction of Phage DNA was carried out by the method of Sambrook and Russel (2001) [32] with some modifications. Briefly, 400 µl of lysate was transferred to 1.5 ml of micro centrifuge tube and was incubated at 56^0C for one hour with proteinase K at a final concentration of 50 µl/ml, 0.5M Ethylene Diamino Tetra Acetic acid (EDTA) at a final concentration of 20 mM and 10% Sodium do Decyl Sulphate (SDS) at a final concentration of one percent. After incubation, the digestion mix in the tube was cooled to room temperature. Afterwards the suspension was extracted with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1 v/v). The phases were separated by centrifugation (Sigma 3K30, Germany) at 13000 rpm for 5 min at room temperature. The upper aqueous layer was transferred to a clean tube using wide-bore pipette. To this, double volume of absolute ethanol and 3 M sodium acetate (pH: 7) to a final concentration of 0.3 M were added, followed by incubation at

room temperature for 30 min. After incubation, the precipitated DNA was collected by centrifugation at 13000 rpm for 10 min at 4^0C. The supernatant was discarded and then added 500 µl of 70% ethanol to wash the nucleic acid pellet. Then centrifuged at 13000 rpm for 10 min at room temperature. The pellet was dried and dissolved in a minimal volume of Tris-EDTA (TE) buffer.

Restriction endonuclease (RE) digestion analysis

The restriction pattern of the Phage DNA was studied using the enzyme, *Eco* RI (Chromous biotech, REN 009A). Enzyme digestion was performed as recommended by the manufacturer. For digestion, each 20 µl digestion solution containing approximately 1 µg of bacteriophage DNA and 1U of RE in reaction buffer, was incubated for 90 min at 37^0C. The RE digested DNA sample were loaded onto 1.2% agarosegel in TBE buffer by mixing loading buffer. Electrophoresis of RE digested samples were carried out along with lambda DNA ladder (Chromous biotech, MAN 05) by applying 50 V current for 2 h. molecular weight of Phage DNA fragment was calculated by plotting distance of migration against molecular weights of marker.

Morphological characterization

Morphological characterization of bacteriophage particles was carried out by using scanning electron microscopy (SEM). The phage particles were fixed in 2.5% glutaraldehyde (pH 7.2) in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4^0C. Remaining Post fixation done in 2% aqueous osmium tetraoxide for 4 h. Dehydration steps were carried out in series of graded alcohols (50%, 70%, 80%, 90% and 100%) and dried to critical point drying with CPD unit. The processed samples were mounted over the stubs with double sided carbon conductivity tape and a thin layer of gold coat over the samples were done by using an automated sputter coater (model: JEOL JFC 1600) for 3 min and scanned under scanning electron microscope (SEM model: JOEL- JSM 5600) at required magnifications as per standard procedures at RUSKA lab's, College of Veterinary Science, PVNR TVU, Rajendranagar, Hyderabad, India.

Results & Discussion

All the 128 samples were positive for *E.coli* by culture method and were subjected to Gram's staining and found Gram negative, coccobacillary rods. The isolates were subjected to the biochemical tests and confirmed them as *E. coli.*

Isolation of bacteriophages

A total of 128 sewage samples from different organized, unorganized and various animal sheds located in and around Tirupati were collected and processed for *E. coli* phages isolation. A total of twenty two (22) *E. coli* phages were isolated from 128 sewage samples. *E. coli* phages were isolated by using tryptic soy agar. The plaques on secondary streaking on *E. coli* mat culture gave the clearing zone around the streak lines Fig.1). The presence of *E. coli* phages were further confirmed by spot test where *E. coli* was grown on nutrient agar and 10µl of isolated bacteriophages against *E. coli* was spotted on different areas of nutrient agar plates and the clear zone around the was observed (Fig. 2). Shukla and Hirpurkar (2011) studied the presence of bacteriophages in sewage material of cattle, pig, goat and poultry and isolated the bacteriophages against two most common environmental bacteria *viz.*: *B. subtilis* and *E. coli* by double agar layer method. Mulani *et al.* (2015) [26] worked on host specific phages from environmental samples and found potential activity of bacteriophages against *E. coli* using spot test, Sundar *et al.* (2009) [41] isolated host specific bacteriophages against *E. coli* from sewage water by using spot test and Maal *et al.* (2015) [23] isolated two novel bacteriophages against *E. coli* SBSWF-27 and *E. coli* PTCC-1399 by using spot test. Olieveira *et al.* (2009) [28] isolated five bacteriophages against *E. coli* from poultry sewage by using spot test method and confirmed their presence by double layer technique (Fig. 3). Manjunath *et al.* (2013) [24] used the sewage sample originated from hospital, domestic, municipal waste and from the waste water treatment plant and isolated phage DMEC-1 against the multidrug resistant *E. coli* by using double agar over layer method and same method was used to isolate phages specific to *E. coli* O157 from waste water treatment plant by Gunathilaka (2014) [19]. Sheng *et al.* (2006) [36] isolated phage SH1 and reported that phage was highly lytic and formed large clear plaques in all *E. coli* O157:H7 strains tested and Nivas *et al.* (2015) [27] found 46 bacteriophages against *E. coli* by processing 10 hospital effluent samples by using double agar layer method. The findings of various scientists mentioned above revealed that spot test and double agar layer method are the two most commonly used methods for the isolation of bacteriophages.

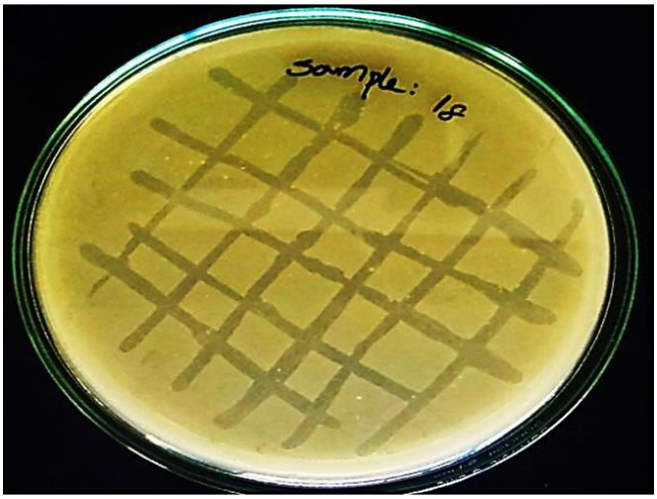

Fig 1: Plaques on secondary streaking on the *E. coli* lawn gave the clearing zone around the streak line

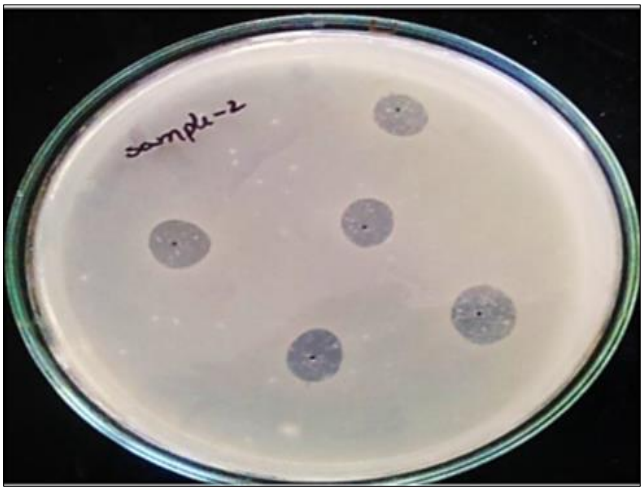

Fig 2: Picture showing lytic bacteriophages against *E. coli*: Spot test

Fig 3: Picture showing lytic bacteriophages against *E. coli*: Double agar overlay method (circular plaques with diameter ranging from (o.1-3mm)

Plaque morphology

The observed plaques were circular with a diameter of 0.1 to 3mm (Fig. 4). Similar to the present findings Bach *et al.* (2003) [4] reported that the phages isolated against *E. coli* from faeces were of same size. Various workers have observed varied plaque diameter varying from (0.1-3.5mm). Shalini *et al.* (2013) [35] and Calderone and Pickett (1965) [9] observed clear plaques of brucellaphage of variable size (0.5-3.5 mm) and Morris and Corbel (1973) [25] observed that the plaques of A422 were clear and varied in diameter from 0.1 to 2.0 mm whereas the plaques of S708 and M51 were of two types i.e. small, turbid plaques, 0.1 to 0.5 mm in diameter and large, clear plaques of 0.5 to 2.5 mm in diameter.

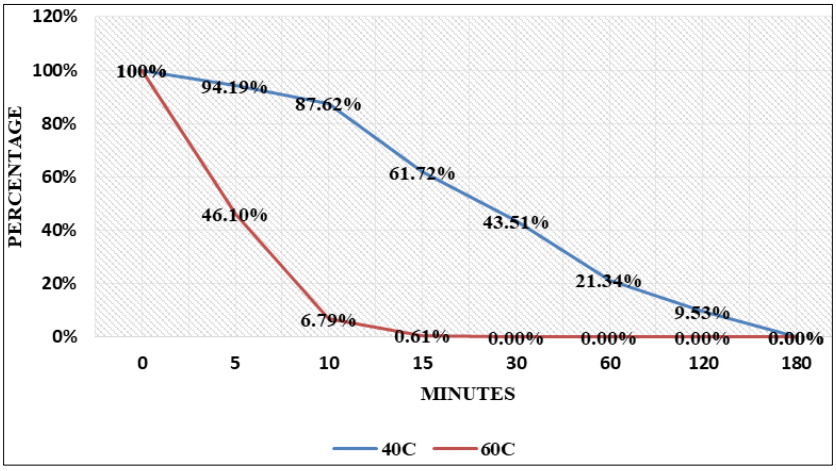

Fig 4: Effect of heat on the activity of bacteriophages (%)

Physico-chemical characterization of isolated bacteriophages

The effect of heat on the activity of phage revealed that at 40^0C phage titre was gradually decreased within three hours, whereas at 60^0C temperature treatment completely inactivated the phage within 30 minutes (Fig. 5). The results obtained in this study were similar to the findings of Al-Mola and Al-Yassari (2010) [3], who reported that the effect of temperature on phage titre was significantly increased at a temperature of 37^0C than at 50^0C and 65^0C. Allue-Guardia *et al.* (2012) conducted heat stability test of phages and reported that at 60^0C there was complete inactivation of phages, Chachra *et al.* (2012) [13] subjected the brucellaphages to different temperatures and reported that there was no growth at 70^0C and 100^0C with in 20 min, Zaman and Arip (2012) [46] characterized the ability of bacteriophages at temperatures ranging between 10^0C-80^0C and reported that phages were stable in a temperature range of 10^0C- 30^0C and became less stable following exposure to 40^0C and 50^0C, Manjunath *et al.* (2013) [24] examined the stability of phages at room temperature and 60^0C and reported that there was 100 fold decrease in titre at 60^0C temperature within 15 min and Shalini *et al*., (2013) [35] reported that the effect of heat on the activity of phages, were gradually deceased at 40^0C within 3 h and at 60^0C temperature, phages were completely inactivated within 10 min. Taj *et al*., (2014) [42] worked on the effect of various ranges of temperatures on T4 bacteriophage lytic activity against *E. coli* and reported that at a temperature regimes of 40^0C, 55^0C and 70^0C the T4 bacteriophages were completely inactivated.

Temperature is one of the most important environmental factor that strongly affects many aspects of the biological systems. One of the important characteristic of the temperature, as environmental factor, is its fluctuation over a wide range of spatial and temporal scales that makes possible as well as limits existence of life in different niches. Influence of temperature upon the biological system is very vivid and it has been observed that evolution of phenotypic traits, species distributions, and extinctions in many cases can be traced to changes in temperature regimes Vale *et al*., (2008) [43]. Present study results are in confirmation with the above findings as during the experiment it was observed that yield of bacteriophage was highly temperature dependent.

The effect of sunlight on activity of phage showed that exposure to direct sunlight gradually decreased the phage concentration and within 3 hours *E.coli* phage titre got reduced (Fig. 6). There are two ways that virus inactivation mechanisms occurs in sunlight, one is through absorption of photons by the virus itself (direct and indirect endogenous inactivation) and the second one is by reaction with reactive species formed by photosensitizers in the water column (exogenous inactivation). The presence of photosensitizing molecules decreased the rate of sunlight mediated inactivation of PV3 signalling that inactivation was dominated by endogenous mechanisms Silverman *et al.*, (2013) [38]. The results obtained in this study were in accordance with Chachra *et al.*, (2012) [13] who worked on brucellaphages and reported that the survivability of phages was gradually decreased from 95.8% to 73% over a 90 min. period and Shalini *et al*., (2013) [35] described the effect of sunlight on activity of phages and reported that there was a gradual decrease in phage concentration within 3 h exposure to direct sunlight.

Exposing the phage to the UV light for 5 minutes caused complete inactivation of bacteriophage (Fig. 7). Inactivation of faecal coliforms and coliphages was mainly by shorter

wavelengths (UV-B) a result consistent with photobiological damage Sinton *et al*., (2002) [39]. The findings of this study were similar to the findings of Allue-Guardia *et al.*, (2012), who worked on Cdtphages and reported that the phages were very sensitive to U.V treatment and Chachra *et al*. (2012) [13] estimated the stability of the phage lysates towards the UV light and reported that the U.V light killed the phages within the first 15 min of exposure. Shalini *et al*. (2013) [35] determined the effect of UV light on the activity of phage and reported that UV light had drastic effect on the phage survivability and also reported that phages were completely inactivated within 3 min.

The effect of SDS treatment on activity of phage revealed that both 1% and0.1% concentrations of SDS completely inactivated the phage within 15 minutes at37^{0}C (Fig.8). SDS is a strong anionic detergent, that can solubilize the proteins and lipids that form the membranes. This will help the cell membranes and nuclear envelops to breakdown and expose the chromosomes that contain the DNA. The results obtained in this study are almost similar to the findings obtained by Chachra *et al.*, (2012) [13], who worked on bacteriophages and reported that complete destruction of the phages was observed within 15 min of exposure to 10% SDS and Shalini *et al*., (2013) [35] determined the stability of the brucellaphages by subjecting them to SDS and reported that complete inactivation of phage was observed within 15 min when exposed to 0.1% SDS.

The effect of phenol treatment on the activity of *E. coli* phages revealed that the phages were completely inactivated by phenol within 30 min and 15 min at 1% and 5% concentration respectively (Fig.9). It suggested that low concentration exponent is associated with inactivation of the phage by an effect on the protein coat of the particle and a high concentration exponent with an effect on its internal structure (Cook and Brown, 1964) [14]. Shalini *et al*. (2013) [35] worked on the activity of brucellaphages against phenol (5% aqueous) and reported that complete inactivation of the phages within 15 min, which is almost similar to the present study.

The effect of chloroform treatment on the activity of *E. coli* phages showed that 5% and 10% concentrations of chloroform completely inactivated the *E. coli* phages within five and three minutes respectively at 37^{0}C (Fig.10). Phage contain lipids as a structure components of their virions and so detection identity to only a few families (Bertani and Bertani, 1986). Sands and lowlicht (1976) [34] showed these lipids are essential for maintaining the virus ability to infect new host. Any disruption of the lipid components will lead to a loss of viability of the virus, lipids are soluble in non-polar solvents such as ether and chloroform, which are capable of extracting and disrupting the lipid components of the phages by interfering with the hydrophobic interactions between lipid molecules. Non-polar solvents are capable of denaturing proteins by disrupting the hydrophobic interaction between proteins, which can also lead to a loss of viability (Camerini-Otera and Franklin, 1972) [10]. Similar to the findings obtained in this study Al-Mola and Al-Yassari (2010) [3] determined the sensitivity of phages to chloroform and reported that chloroform completely inactivated the phage within 5min, Shalini *et al*. (2013) [35] determined the effect of chloroform on the stability of phages and reported that complete inactivation of phages within 5 min, whereas Manjunath *et al*. (2013) [24] reported that there was no decrease in the Phage titre in chloroform treated phages after 4 and 24 h incubated at room temperature.

Exposure of phage to 40% formalin up to 15 minutes causes complete inactivation of phages (Fig.11). The destructive action of formalin may be attributed by its alkylation of protein and nucleic acids (De Benedictis *et al.*, 2007). The results obtained in this study were in accordance with Shalini *et al*. (2013) [35], who reported that complete inactivation of phages within 15 min. when exposed to 40% formalin.

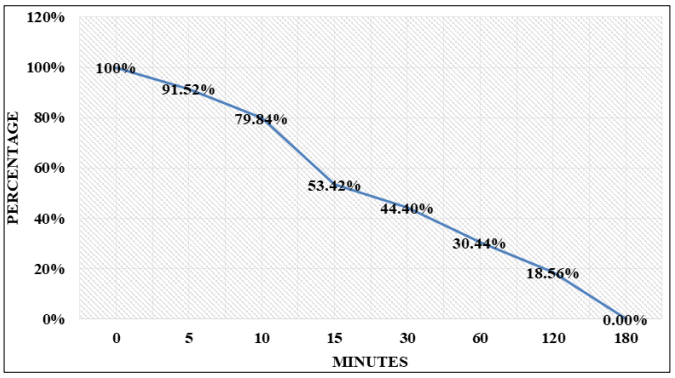

Fig 5: Effect of sunlight on the activity of bacteriophages (%)

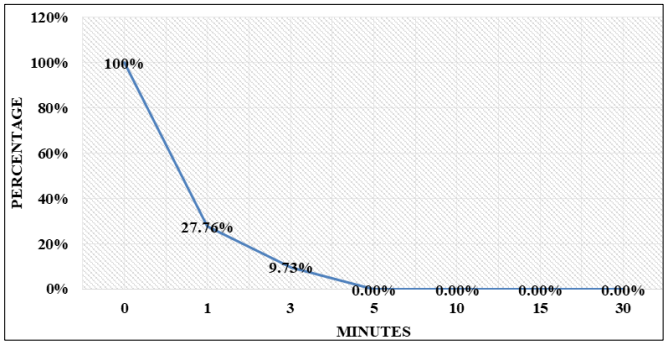

Fig 6: Effect of UV light on the activity of bacteriophages (%)

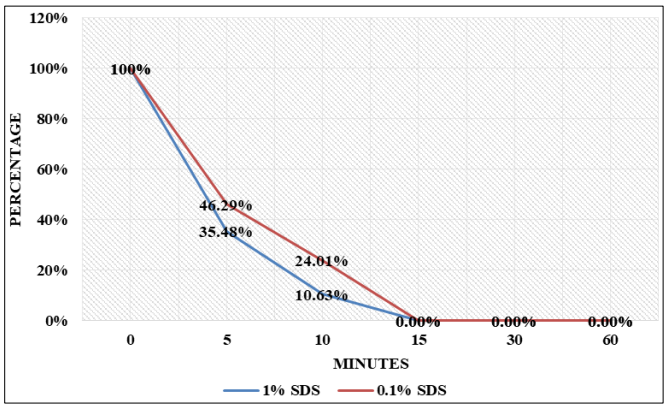

Fig 7: Effect of SDS on the activity of bacteriophages (%)

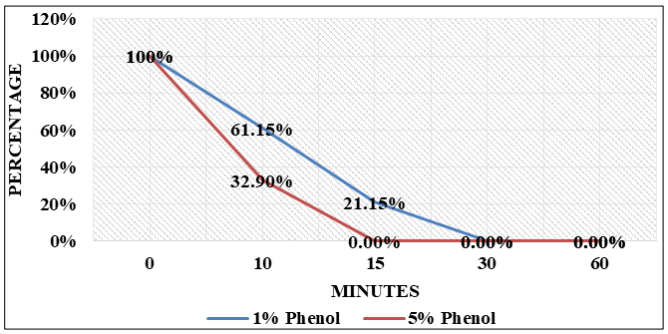

Fig 8: Effect of Phenol on the activity of bacteriophages (%)

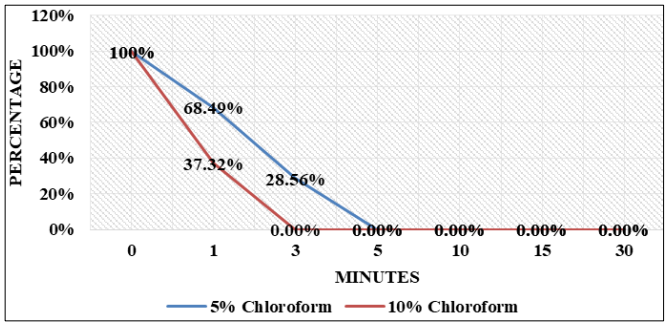

Fig 9: Effect of Chloroform on the activity of bacteriophages (%)

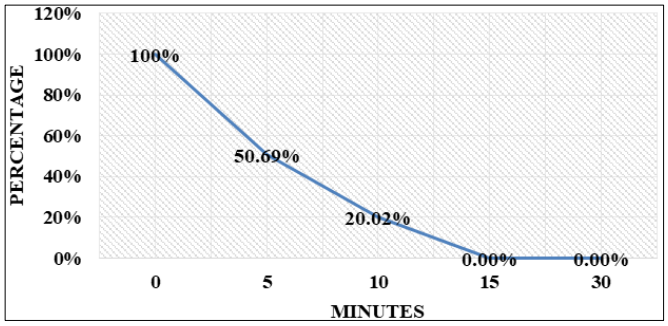

Fig 10: Effect of Formalin on the activity of bacteriophages (%)

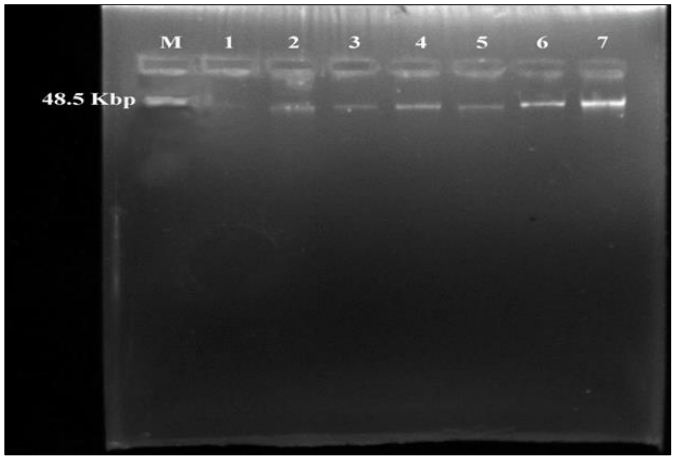

Fig 11: Determination of molecular weight of E. coli phage nucleic acid

RE digestion analysis

Restriction enzymes such as *Eco* RI recognizes specific sequences of DNA and cleaves the phosphor diester bond on each strand at that sequence. After digestion with a restriction enzyme, the resulted fragments can be separated by agarose gel electrophoresis and their corresponding sizes can be estimated (vlab.amrita.edu., 2011). In the present investigation phages appear to have similar profiles of the nucleic acid fragments generated by digestion of their DNA with *EcoRI* (Fig.12).

The findings obtained in this study were in accordance with Goodridge *et al.* (2003) [11], who performed restriction enzyme analysis with four enzymes and confirmed the phages isolated against *Escherichia coli*, Jamalludeen *et al.* (2009) [20] determined the genome size of isolated phages EC-Nid1 and EC-Nid2 by using Restriction enzymes, Bao *et al.* (2011) [6] performed restriction enzyme analysis with *Eco RI* and *Hind III* to digest genomes of the bacteriophages and Jamalludeen (2012) [21] isolated Phages EC-NJ4 and EC-NJ7 against *Escherichia coli* and also reported that the two phages appears to have similar profiles of the nucleic acid fragments generated by restriction enzyme digestion.

Morphological characterization of phages by SEM

The SEM observations of phage revealed that the phage had icosahedral head with short tail (Fig. 13). It was found that based on morphology the phage exhibited non-contractile tail and belong to the order *Caudovirales* and family *Podoviridae*.

Activity of bacteriophages against target bacteria

To detect the activity of phages against target bacteria *in vitro* studies were conducted by taking the sewage sample and isolated phages were inoculated in to the same and the samples were analysed at every 5 hour interval. The results of this study revealed that there was complete inhibition of *E. coli* after 10 hours of incubation (Table.1). The results obtained in this study were almost similar to the results obtained by Maal *et al.* (2015) [23] reported for the first time on *in vitro* isolation and identification of two novel bacteriophages that have lytic effect on *E. coli* PTCC1399 and *E. coli* SBSWF27 strains as well as coliform population of Isfahan municipal wastewater and further he reported that there was a 22-fold reduction in coliform load in wastewater with coliphages incubation for 2 h and Mulani *et al.* (2015) [26] worked on potential activity of bacteriophages against target bacteria by inoculating bacteriophages in actively growing host and reported that there was 100% reduction in *E. coli* and *Salmonella* count within 9 h.

Table 1: Activity of bacteriophages against *E. coli* isolated from sewage samples

Sample No.	*Escherichia coli* count at							
	0 h		5 h		10 h		15 h	
	T1	T2	T1	T2	T1	T2	T1	T2
1.	13	21	141	14	211	00	65	00
6.	11	18	138	13	189	00	56	00
11.	7	11	82	8	122	00	29	00
13.	12	19	143	17	204	00	65	00
16.	15	24	169	19	275	00	73	00
18.	11	20	116	12	201	00	62	00
23.	13	19	137	13	228	00	67	00
26.	12	21	134	14	212	00	65	00
29.	8	12	94	11	145	00	38	00
31.	11	21	131	13	195	00	53	00
33.	9	17	102	11	156	00	49	00
42.	14	23	159	21	247	00	68	00
51.	15	27	163	27	286	00	79	00
53.	9	14	98	13	165	00	48	00

55.	12	23	139	17	217	00	71	00
58.	8	15	91	12	142	00	41	00
64	7	13	75	9	121	00	37	00
82.	7	16	71	10	128	00	29	00
95.	13	26	146	16	236	00	59	00
107.	11	16	118	13	193	00	51	00
119.	15	29	171	31	267	00	81	00
127.	10	14	109	11	183	00	52	00

The *in vitro* studies carried out to know the activity of bacteriophages against *E. coli* determined that these phages readily killed the target bacteria. So use of bacteriophages for reducing pathogenic bacteria in sewage along with other standard methods like primary and secondary treatment could be considered as an effective and simple alternative for replacement of costly instruments and establishment of the old wastewater treatment plants Maal *et al*., (2014) [22]. Phage mediated bacterial mortality has the capacity to influence treatment performance by controlling the abundance of pathogenic bacteria (Periasamy and Sundaram, 2013) [29]. Many wastewater treatment plants aim for the complete pathogen removal during treatment by using disinfectants like chlorine which can harm the environment. Hence development of cheapand eco-friendly approaches is necessary. Pathogen specific phages isolated from sewage have the potential to eliminate the dreadful pathogens (Periasamy and Sundaram, 2013) [29].

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