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Research Paper

ISOLATION, CHARACTERIZATION AND RECOVERY OF COLIPHAGES FROM SEWAGE SOURCE

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Abstract

In bacteriophage research, host specific coliphage study was undertaken considering six aspects. (i) Identification of *E. coli* strains (*E.coli* from isolated from sewage source) using standard IMVIC test. (ii) Establishment of coliphages Wild type (sewage source) plaques on *E.coli* lawn on Tryptose soya agar (TSA). (*E.coli* isolated from sewage source) (iii) Effect of various adsorbents and elluent buffers recovery of phage titer on *E.coli* on TSA plates. (iv) Coliphage adsorption assay with Aluminum Chloride, Magnesium Chloride and Calcium Chloride as adsorbent and MaclLvaine buffer as elluent on Nutrient agar, TSA, M-FC agar plates (v) Coliphage assay using application of sterilized Soil extract as adsorbent and MaclLvaine buffer as elluent on TSA plates. (vi) Efficiency of coliphages recovery using Aluminum Chloride as adsorbent and various buffers as elluent i.e. MaclLvaine buffer, Beef extract, Tris buffer, Glycine buffer and Casein buffer. Uncertainty in bacteriophages numbers and recovery part on bacterial lawn is subject to state of the art. Coliphages plaque serial no. 17 have shown 10900/ml plaque count on EC-5 *E.coli* (sewage source) TSA plates. When Coliphage was concentrated with Aluminum Chloride as adsorbent and MaclLvaine buffer as elluent produced 147 plaques/ml on TSA and 278 plaques/ml on M-FC Medium after 48 hours. Sewage filtrate (1:10) sample (10ml-Sewage Filtrate added to 90ml dechlorinated tap water) with $CaCl_2$ as adsorbent /MclLvaine buffer as elluent was shown 800 plaques/ml on TSA plates. It is found effective in concentrating coliphages. Soil Extract as adsorbent of coliphage in plaque count evaluation indicated that soil is poor adsorbent of coliphages due to presence of organic matter and only 14 plaques/ml was observed as compared to 85 plaques/ml $CaCl_2$ as adsorbent /MclLvaine buffer as elluent on TSA plates. Coliphage recovery 70% was observed with host E.coli lawn on TSA plates.

Key words: Coliphages, *E.Coli*, IMVIC test, phage titer, Efficiency of phage recovery.

INTRODUCTION

Large quantity of wastewater ultimately discharged through sewerage system into the flowing stream as the effluent disposal option in most of the cities in India. *Escherichia coli* are known as Indicator bacteria for presence of pathogenic bacteria. Pathogenic bacteria survive in waste water cause aquatic pollution and deteriorate water quality. Viruses and bacteriophages are require specific host for survival and spread through air, water and direct contact. The possibilities of naturally occurrence of bacteriophages are prime concern of removing fecal coliform & other enteric origin bacteria i.e. *Serratia* sp., *Camphylobacter* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Streptococcus* sp., *Bacillus* sp., *Salmonella* sp., in polluted water sources. Viruses infect bacteria which d'Herelle called coliphages classified based on morphology of capsids and nature of nucleic acid and belong to six category [1] (i) Myoviridae: Capsid with contractile tails with 2ss DNA classified as T2,T4, and T6 (ii) Siphoviridae: Capsid with noncontractile tails with 2ss DNA λ and T5 (iii) Podoviridae: Capsid with short contractile tails with 2ss DNA, T3 and T7 (iv) Microviridae: Capsid with large capsomere and Icosahedrons with 12 apical capsomere with 1ss DNA –φ X 174 phage (v) Leviviridae: Small capsomere with 1ss RNA - Group1: F2, MS-2, R-17, JP 501; Group 2: GA,DS,TH1, BZ13; Group 3: QB, VK, ST, TW 18; Group 4: SP, F1, TW 19, TW 28 (vi) Inoviridae: filamentous phages with 1ss DNA SJ2, fd, AF-2,M13. F-RNA coliphages were rarely detected in humans and cattle faeces but more often in faeces from pigs and adult chickens [2]. Viruses and consequently bacteriophages are removed from water by adsorption to particles, sedimentation and filtration through porous matrixes either soil or manmade filters [3]. Findings illustrated that surface water spiked with waste water infiltrate showed at different pore water velocities (PWV) at different migration distances in slow sand filtration [4]. Similarly comparison of selected media in coliphage assay including Nutrient broth (with 0.05% sodium deoxycholate) and lactose broth (with 0.05% sodium deoxycholate) and lactose broth and subsequently plating on EC medium, Nutrient agar, TSA, were conducted for enhanced recovery of coliphages from a eutrophic lake receiving treated sewage effluent [5].

Non-variable, Electrostatic surface properties of the viruses are exploited to enable adsorption at their iso-electric point of pH 3.5. The similar approach may be applied for isolation of coliphages from domestic sewage [6] [7]. Bacteriophage binding and

adsorption to host cell and penetration is probably due to electrostatic interactions and is influenced by pH and the presence of ions such as Mg^{2+} and Ca^{2+}.

In contrast, presence of viral indicators in water classified water suitable for bathing by European Directive and alerts. the suitability of its 15 beaches for bathing throughout each season was assessrd in 1996. The ratio between SOMCPH and *E. coli* somatic coliphages (SOMCPH) and F-specific RNA bacteriophages (FRNAPH) enabled the study of the link between bacterial and viral indicators as well as the analysis of the ratios between both types of indicators in waters with different level of pollution. Study indicates at low level of pollution (*E.coli* <100 MPN/100ml) i.e *E.coli* numbers are lower than those of SOMCPH while *E. coli* outnumber SOMCPH at higher levels of pollution (*E.coli* >100MPN/100ml) [8]. Study of temperate and virulent lambda coliphages using markov chain stochastic model shows that there were stochastic switch from lysogen to lytic growth in experiments [9].

MATERIAL AND METHODS

Bacteriophage assay required the following materials: filtration assembly and Filter holder, Autoclave, pH meter, beakers, laboratory balance, Graduated cylinders, micropipettes, and Millipore filter papers (0.22µm/0.45µm pore size), Hydrochloric acid (HCl-1N), Sodium hydrochloride (NaOH- 1N),

Polyelectrolyte's as Elute solution for adsorption of coliphages: (i) Magnesium Chloride ($MgCl_2$) 243.8mg/100ml to prepare 1200 ppm solution (1.2mM), (ii)Calcium Chloride ($CaCl_2$) 184mg/100ml to prepare 1200ppm (1.2mM), (iii) Aluminum Chloride ($AlCl_3$) 6.03mg/100ml to prepare (0.05mM).

All the above electrolytes solutions pH adjusted to 3.5 and were used for differential adsorption of bacterophages to 0.22 µm filter paper [10].

Buffers for elution of bacteriophages from FP:

McllLvaine buffer as elluent: Stock solution (i) Disodium Hydrogen Phosphate ($Na_2HPO_4.2H_2O$),15.468g/100ml (0.87M); Stock solution (ii) Citric Acid solution 12.5%; 10ml of stock solution (i) was added to 100ml deionized water and then 3g of beef extract was dissolved in stock solution (i) and pH 7.1 was adjusted with citric acid solution stock solution (ii).

Beef Extract solution: 3% solution of beef extract (Diffco make) (pH 6.0); Tris buffer: 0.2 M solution contains (24.23/100ml of Tris) (pH 9.0); Glycine buffer: 0.2 M solution

(15.01 g/1000ml of Glycine) (pH 9.5); Casein buffer: 2% solution (pH 9.0); all buffer solutions was adjusted with 1N NaOH and 1N HCl solutions.

E.coli strains were isolated for sewage source for coliphage assay from the NEERI campus nalha at Nagpur. *Escherichia coli* was maintained in the nutrient agar slants and kept at -4^{0}C in the refrigerator Whenever required inoculated in tryptone broth for experimental set studies.

Culture media: *Escherichia coli* were grown on M-Endo agar medium and M-FC agar medium,. Selected colonies were enriched in tryptone broth and concentration of E. coli was determined at 520 nm using spectrophotometer, (Spectronic -20). Nutrient agar and TSA medium were used for bacteriophage plaque assay.

Nutrient agar: Peptone 5.0 g/l, beef extract 3g/l, Sodium chloride 5g/l, Agar 20g/l dissolved in 1000ml distilled water pH-adjusted to 6.8 at 25^{0}C. 2,3,5 triphenyl tetrazolium chloride (TPTZ) 1% (W/V) in ethanol added to NA at 45^{0}C to enhanced plaques visibility.

Tryptone Soya Agar medium(Hi-Media Lab) : Tryptone 17.0g, Soytone 3.0g, Dextrose 2.5g, Sodium chloride (NaCl) 5.0g; K2HPO4-2.5g; Extrapure agar 2.0%, Deionized water 1L. pH should be adjusted to 7.3 at 25^{0}C.2, 3, 5 triphenyl tetrazolium chloride (TPTZ) 1% (W/V) in ethanol added to NA at 45^{0}C to enhanced plaques visibility.

Garden soil preparation:

1 g Garden soil suspended in 9ml distilled water was sterilized in autoclave on 121^{0}C, 15 psi, for 15 minutes (pH to 7.4 adjusted before autoclaving). Soil was then stirred in 9ml of MclLvaine buffer for 30 minute.

RESULTS AND DISCUSSION

Coliphage assay: E.coli was isolated from sewage source diluted $10^{-4,}$ and 10^{-5} dilution, and passed through 0.45µm filterpaper and filterpaper was then placed on M-Endo agar media. Red colonies with metallic shine were sub cultured on M-FC agar medium. Gram negative, and all indole and MR test positive *E. coli* strains of IMVIC test were selected for coliphage detection purpose.

In first set, *E.coli* culture broth was incubated 42 to 48 hours to acquire coliform numbers 4.04X10^7/ml corresponding to optical density 0.438 and for another set corresponding to coliforms count 6.46x10^6/ml to optical density 0.383 at 520nm and broth cultures were used for performing plaque assay. In general coliphages were

recovered from different water sources using plaque counts method. The isolation procedure host specific coliphages is done by preparing type cultures of *E. coli* lawn on or Nutrient agar, M-FC agar and TSA very much similar to more cumbersome procedure adopted for concentration of animal virus i.e. isolation could only be possible on animal cell lines e.g. Buffalo green monkey kidney cell line (BGMK cells) a continuous cell line for enteric virus.

Electronegative nature helps in concentrating of virus or bacteriophages on Millipore filter papers. The electronegative filter are composed of either cellulose acetate or Glass fiber filter paper as support media and Coliphages or virus get eluted most efficiently in the presence of multivalent cations Mg^{2+}, Ca^{2+}, and Al^{3+} at low pH usually 3.5 and adsorbed on filter paper coliphage then concentrated with organic nature buffer (elluent) at higher pH ranges 6.0 to 9.5. Beef extract and mclLvaine buffer were used for elution of coliphages and subsequently plated on the lawn of *E. coli*. *E. coli* isolated from sewage sources (named as EC1 to EC5) (Table-1). T even (based on plaque shape) coliphages was enriched with the *E. coli* for plaque count on the lawn of EC-1, EC-5. Plaque counts reported from water samples 1 to 17 enriched with E. coli. Nutrient broth with phage titer plated with sewage source E. coli and E. coli on extra pure agar. The coliphage plaques were varied from 120 to 10900/ml (Table-2).

2^{nd} experiment set was conducted for coliphage isolation from different sources. coliphage detection was conducted from distilled water, propogated coliphage and raw sewage effluent of 10^{-3} and 10^{-4} dilution. Elute solution and elluent mclLvaine buffer and beef extract used in concentrating coliphages. Nutrient agar M-FC agar and TSA were used in coliphage detection assay. No plaques were observed with sterilized distilled water and propogated phage. While Variation in phage counts on plating 10^{-4} diluted effluent showed 6-8 coliphage plaques and increased coliphage counts 86-134 were detected in 10^{-3} diluted effluent on nutrient agar and TSA (Table-3).

Different set of concentration, and plating methods for coliphage transfection on E.coli strains was studied. (i) Sterilized distilled water and dechlorinated tap water (control) (ii) diluted sewage filtrate (iii) diluted sewage filtrate with dechlorinated tap water. Coliphages were eluted with $CaCl_2$ and $MgCl_2$ as adsorbent and mclLvaine buffer and beef extract as elluent solution. The coliphage concentrations in filtrate were determined. Result showed that (i) control with dechlorinated tap water and $MgCl_2$/BE; control with dechlorinated tap water and $CaCl_2$/ mclLvaine buffer were indicated no

coliphages after plating on TSA (ii) sewage filtrate 10^{-2} diluted with distilled water was concentrated with $CaCl_2$/ mclLvaine buffer indicated high coliphage counts ranging from 26 to 800/100ml on TSA and sewage filtrate 10^{-2} diluted with dechlorinated tap water was concentrated with $CaCl_2$/ mclLvaine buffer showed low coliphage counts were ranged from 5 to 51/100ml on TSA (iii) sewage filtrate 10^{-2} diluted dechlorinated water was concentrated with $MgCl_2$-Beef extract and $MgCl_2$ and mclLvaine buffer, coliphage counts ranged from 1-26/100ml on TSA dechlorinated tap water (Table-4).These type of plaques were characterized belong to two class (i) tail less phages with large capsomeres icosahedrons with 12 apical capsomeres such as Øx174 (ii) Single stranded RNA phage identified as f2 (tail less with small capsomere) variation to observed plaque morphology subdominant type plaque morphology of coliphage T-even phages (DS-DNA phages) with six side outline complex having contractile tail assembly with head [11]. Although considering the fact for exploiting the possibility of using sterilized Garden soil as adsorbent for coliphages, based on surface soil layers contain large amount of Ca^{2+} and Mg^{2+} ions may contribute to adsorbent for phages, was performed for concentration of coliphages. Coliphage detection using Soil extract (4ml soil extract of soil dry weight weight 1333.6mg or 1.500mg) as elute and mclLvaine buffer as elluent was performed. Similar dilution of sewage effluent was eluted using $CaCl_2$ 200ppm elute and mclLvaine buffer as elluent to know the effectiveness of soil was also conducted. The replicate of plaque assay was performed using *E. coli* culture on TSA incubated at 44^0C at 24-48 hours. $CaCl_2$ and sterilized soil as adsorbent were retained 37 to 85 plaques/ml and 4-14 plaque/ml of coliphages on TSA (Table-5). Poor adsorbent of coliphage was may be due to organic matter of soil. Sand bed and magnetite organic flocculation method so far developed for the concentration of coliphages from waste water effluents and polluted lake water and high percentage (68 to 100%) recovery of coliphage from sewage effluent was achieved by above method [12].

In present study, recovery of coliphages was also performed. Coliphages were detected in diluted 10^{-6} to 10^{-8} sewage effluent samples. Coliphages from sewage effluent was recovered using $AlCl_3$ elute and MclLvaine buffer elluent entrated with MclLvaine buffer, Beef extract, Tris buffer , Glycine buffer and Casein buffer on *E.coli* lawn, Concentrated sample were plated on the M-FC agar. 1ml *E.coli* culture broth, 1ml coliphage suspension and 7.5 ml of molten (45^0C) plated on M-FC agar. Initial coliphage

concentration was 1514/ml in the inoculums was concentrated using different buffers. Recovery of phages was indicated 1 plaque in 10^{-8} diluted sample to maximum representative plaque count 278 plaques in 10^{-6} diluted sample plated on M-FC agar lawn. Plaque morphology shows the clear plaques on E. coli culture plates (Table–6). In presence of salts, coliphage cause *E. coli* lysis. Lowest recovery of coliphages 320/ml (20.11%) was observed with Casein buffer than as highest recovery of coliphages 1120/ml (70.39%) with MclLvaine buffer after plating recovery of coliphages on EPA Figure -1). The salient part is that phages adsorbed its host bacteria in presence of salt concentrations has high efficiency of plating while on salt free agar has low efficiency of plating.

The phages molecular weights were determined using optical mixing spectroscopy and sedimentation coefficient for T4, T5, and T7 were then calculated to be 192.5± 4.0 and 50.4±1.8 from the percentage DNA in these phages (in Million Dalton) and mol.wt. of T4, T5 and T7 DNA were found to be (in Million Dalton) 105.7±3.8, 67.3 ±3.1 and 25.8±1.0 [13].

Coliphages in sewage where they arrived, presumably after passing through the Gastrointestinal (GI) tracts of animals inhabited commensally by coliform bacteria. Susceptibility of host bacteria are seem to be most likely barrier to establishment of new coliphage infection in the mouse gut. While evaluating water quality, it is noticed that coliphages showed strong association with indicator bacteria and Enterovirus, but weak association with other enteric viruses [14] . MS2 phase was observed below the threshold of detection in reverse osmosis process (RO) although Microfiltration membranes reduce less than one log unit [15].

Using Simulation model, optimal latent period especially at lower bacterial densities is lower where phages dispersal time vary in natural phage population. Host density itself is likely to be variable, which should affect optimal latent period. Second host quality is likely heterogeneous and true for population of phage sensitive and partially resistant bacteria (e.g. Coliphage T2) and for polyvalent phages (strain that can infect multiple host species). Such heterogeneity in patch quality should also affect the optical latent period. More severity was observed in the presence of Enterovirus, Rotavirus and Astrovirus. Coliphages and indicator bacteria recognized as being responsible for diarrheal disease in young children with a world wide mortality rate of 600,000 per year. Astrovirus is also considered one of the most important agents of viral

gastroenteritis. These enteric viruses has characteristics, which allow them to survive in the environment for longer periods of time, and tolerate changing environmental condition.

The liquid colorimetric presence-absence (LCPA) assay was developed to avoid the problem of false positive plaque like areas and to shorten the time required for detection. LCPA method was determined to be as sensitive as the APHA method (1989) and the LCPA yielded results in 45 hours and 24 hours. Because β-galactosidase is a large oligomeric protein it cannot diffuse through the cell envelope of E.coli. Therefore, β-galactosidase in the medium can only come out from lysed or damaged cells [16]. In LCPA assay cultures were filtered and 1 ml sample of the filtrates added to 9 ml of Z buffer to assay β-galactosidase activity. Chlorophenol red β-galactoside (CPRG) was added as a chromogenic substrate for β-galactosidase to form purple colored of chlorophenol red. Thus any reaction that turns purple containing β-galactosidase while any reaction that remains yellow does not contain coliphages. β-galactosidase release requires coliphage induced lysis resulting in β-galactosidase activity as a reflection of coliphages in a sample. When using a concentrated water sample, LCPA method detects as few as 2 plaque forming units/liter as compared to the APHA's (1989) sensitivity to 5 PFU/100ml. In field trials of LCPA and APHA assays, 90 samples of tap, spring, creek and waste water samples, LCPA produced by 3.3% (3 of 90) false negative results compared to the APHA method.

Table-1: Characterization of *Escherichia coli* isolated from sewage effluent Nalha at NEERI, Nagpur

	Escherichia coli	IMVIC Test			
		Indole Test	Methyl Red	Vauges prauskaur	Citrate utilization
1	EC1	+	+	-	-
2	EC2	+	+	-	-
3	EC3	+	+	-	-
4	EC4	±	+	-	±
5	EC5	+	+	-	-

Table 2: Isolated from sewage effluent Nalha at NEERI, Nagpur and count on *E. coli* lawn of TSA

Sr. No.	Water Samples (serial numbers) enriched with *E.coli* in Nutrient broth for Coliphages	Enrichment and and subsequent plating after 6 hours with *E. coli* isolated form sewage effluent stream at NEERI, Nagpur	Coliphage plaque count/ml on TSA
1	1	EC-1	120
2	2	EC-1	238
3	3	EC-1	4920
4	14	EC-1	3310
5	16	EC-1	3870
6	17	EC-5	10900

Table-3: Coliphage plaque counts with E.coli using $AlCl_3$ (adsorbent and MclLvaine buffer (elluent) on Nutrient agar, TSA and M-FC agar

S. No.	Culture media	Inoculums	Plaque /ml	
			1st day	2nd day
1	Nutrient agar	1ml (Distilled water)	Nil	Nil
2	TSA	1ml(10^{-3}) Propagated phage	Nil	Nil
3	Nutrient agar	1ml (10^{-4})	6	8
4	TSA	1ml (10^{-4})	6	6
5	Nutrient agar	1ml(10^{-3})	86	116
6	TSA	1ml (10^{-3})	120	134
7	M-FC agar	1ml (10^{-8})	Nil	1
8	M-FC agar	1ml (10^{-8})	4	9
9	M-FC agar	1ml (10^{-7})	36	39
10	M-FC agar	1ml(10^{-7})	54	58
11	M-FC agar	1ml (10^{-6})	217	253
12	M-FC agar	1ml(10^{-6})	258	278

Density of *E.Coli* Inoculums in Culture broth 0.383 and 0.438 was equivalent to $6.45x10^6$/ml and 4.04x107/ml at 520nm under UV/Visible Spectrophotometer.

Table-4: Coliphage plaque counts with *E.coli* using $CaCl_2$ and $MgCl_2$ (adsorbent) and MclLvaine buffer/ Beef extract (elluent) on TSA

S. No.	Sample source	Adsorbent/ Elluent	Plaque/100ml of sample on TSA
1	Distilled water(Control)	$CaCl_2$/MclLvaine buffer	Nil
2	Sewage filtrate (1:10)	$CaCl_2$/MclLvaine buffer	26
3	Sewage filtrate (1:10)	$CaCl_2$/MclLvaine buffer	800
4	Distilled water(Control)	$MgCl_2$/ MclLvaine buffer	Nil
5	Dechlorinated tap Water	$MgCl_2$/ MclLvaine buffer	5
6	Sewage filtrate (1:10)	$MgCl_2$/ MclLvaine buffer	51
7	Distilled water(Control)	$MgCl_2$/Beef Extract	Nil
8	Sewage filtrate (1:10)	$MgCl_2$/Beef Extract	26
9	Sewage filtrate (1:10)	$MgCl_2$/Beef Extract	5

Sewage filtrate (1:10)-(10ml-SF added to 90ml Distilled water)

Density of *E.Coli* Inoculums in Culture broth 0.383 and 0.438 was equivalent to $6.45x10^6$/ml and $4.04x10^7$/ml at 520nm under UV/Visible Spectrophotometer

Table-5: Coliphage plaque counts with *E.coli* using sterilized soil extract as adsorbent and MclLvaine buffer (Elluent) on TSA

S. No.	Sample sources	*E.coli*	Adsorbent/Elluent	Plaques/ml on TSA
1	Distilled water(Control)	$1.16x10^6$/ml, (inoculums-2ml)	$CaCl_2$/MclLvaine buffer	Nil
2	Sewage filtrate (1:10)	$2.0x10^5$/ml (inoculums-2ml)	Filtered soil extract /MclLvaine buffer	4
3	Sewage filtrate (1:10)	$1.16x10^6$/ml, (inoculums-2ml)	$CaCl_2$/MclLvaine buffer	37
4	Sewage filtrate (1:10)	$1.16x10^6$/ml, (inoculums-2ml)	Filtered soil extract/MclLvaine buffer	14
5	Sewage filtrate (1:10)	$2.0x10^5$/ml (inoculums-2ml)	$CaCl_2$/MclLvaine buffer	85

Sewage filtrate (1:10)-(10ml-SF added to 90ml Distilled Water)

Table-6: coliphage recovery using different buffer elluent and host *E.coli* on TSA

S.No.	Elluent buffer	Coliphage titer /ml in Inoculums	Coliphage/ml after plaque count	Percentage recovery
1	MclLvaine buffer (pH 7.1)	1600	1120	70%
2	Beef Extract (pH 6.0)	1600	360	20.50%
3	Tris buffer (pH 9.0)	1600	320	20%
4	Glycine buffer (pH 9.5)	1600	440	27.50%
5	Casein buffer (pH 9.0)	1600	320	20%

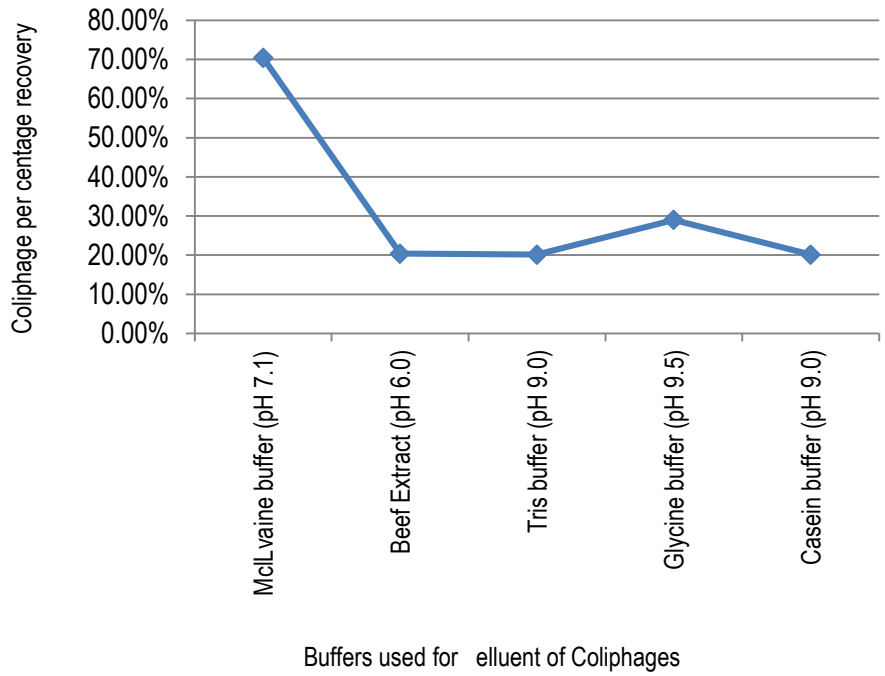

Figure-1: Coliphage recovery using different buffer (elluent) and host E. coli on TSA

CONCLUSIONS AND ACKNOWLEDGEMENTS

Surface water from the reservoirs is utilized for domestic purpose after suitable water treatment in the water treatment facility in the Cities. *E.coli* is usually infested by host specific coliphages. Coliphage increases fallowing the single step growth curve. Furthermore potential risk to the exposed population, especially in developing countries, considering that recycled water at randomly selected sampling points has been associated with the presence and re-emergence of water borne diseases worldwide. Although it is important to consider microbial water quality in terms of water use and not possibly to establish a direct relationship between epidemiological and environmental data.

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