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Bacteriolytic Activity of Coliphages on Diarrhea Associated *E. coli*

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Abstract
There has been an alarming increase in drug-resistant strains of diarrheagenic Escherichia coli (DEC) in developing as well as developed countries. Several cases of antimicrobial resistance in DEC have been observed in different parts of the world as a result, there has been a renewed interest in alternative antimicrobial treatments, including bacteriophages. This study was conducted to isolation and characterization of a lytic coliphage from sewage water capable to infect a variety of multidrug resistance DEC strains isolated from children suffering diarrhea, as first step to further usage a lytic coliphage in future.in this study, a coliphage was isolated using spotting method and titrated, using agar overlay technique. The host range of coliphages was assessed on a lawn of E coli bacteria. This study included determination of the latent periods and burst size of coli phage then determines the stability of coliphages to physical and chemical condition (temperature, pH and sunlight exposure).The results shown that, five phages isolate (A, B, C, D and E) were exhibiting a potent lytic activity with clear plaques (1-4mm in diameter). Fifty percent of the E coli strains were infected by phage isolates. It seems, very likely, that the coliphages belonging to 3 different groups (1, 2 and3). The phage growth cycle with a detected latent period of 20 min, a burst size of 160 plaque forming units per infected cell, it was found that the phage could survive at varied pH conditions with reduction in its numbers. A temperature of above 60°C and direct sunlight beyond 8 days was found to be deleterious for survival of the phage.
Keywords: key words, coliphages, *E. coli*, diarrhea

1. Introduction

Bacterial viruses, known as bacteriophages or phages (from the Greek phagein, 'to eat') (Carter and Saunders, 2007) Bacteriophages are bacterial viruses that only infect and multiply within their specific hosts. Host specificity is generally found at strain and, species level, or, more rarely, at genus level. This specificity allows for directed targeting of dangerous bacteria using phages. Bacteriophages are the most numerous form of life on Earth; ten times more numerous than bacteria (Hagens and Loessner, 2010). They can be found in all the environments where bacteria grow: in the desert, hot springs, the North Sea, and polar inland waters (Jończyk *et al*., 2011; Sundar *et al*., 2009). These phages are important in order to regulate bacterial abundance and its distribution (Carter and Saunders, 2007). Bacteriophages that infect E. coil sometimes are referred generally as coli phage. In other words, coliphage can replicate only within coliform cells. Phage must attach to a receptor on the surface of a bacterial cell in order to initiate an infection. This interaction between the phage and receptor is very specific. a given phase type only will bind to a specific receptor molecule. Thus, all phage are not alike (Al-Mola and Al-Yassari, 2010). Two functional types of coliphages exist in the environment: male-specific (F+) and somatic coliphages. F+ coliphages infect their bacterial hosts by attachment to the F-pilus of the cell. Therefore, F+ coliphages only infect hosts that contain the F+ plasmid and can produce F-pili. Somatic coliphages infect bacterial hosts by direct attachment to cell walls. Coliphages have been suggested as indicators for the presence of enteric viruses in water because they have similar physiologically characteristics to some human enteric viruses, and are often found in the intestinal tract of humans and animals (Rodrígueza *et al*., 2012).They classified into five families: Myoviridae, Siphoviridae, Podoviridae and Microviridae. All families are found in the sewage, although Myoviridae and Siphoviridae are the most abundant (Muniesa *et al*., 2003). The sensitivity of a host strain to a particular bacteriophage is usually measured by the diameter of the lytic zone around the spot of a small volume of phages suspension onto a lawn of host bacteria. The size of this zone indicates sensitivity of the host bacteria to the tested bacteriophage (Adams, 1959) (Muniesa *et al*., 2003).

2. Material and Methods:

2.1 Isolation of pathogens

The study was carried out on children suffering from bacterial diarrhea. (150)Stool samples, from those children aging from 20 days to 60 months, were cultured on MacConKey agar as primarily isolation for the presence of Enterobacteriaceae through incubation at 37^0C for 24 hr.. Colonies became visible on media after 24hr. of incubation. Finally select colonies for purification and identification. Isolates were identified depending on morphological on selected media and biochemical tests as compared with identification scheme described by (Mahon 2007) then selected E coli isolates only were to use in this study.

2.2 Demonstration of coliphages

2.2.1 Preparation of bacteriophages Suspension: A bacteriophage suspension was prepared from sewage sludge. Sludge (10 ml) were transferred to a sterile 25 ml centrifuge tube and centrifuged at 2000 rpm for 10 min. After centrifugation, the supernatant was aseptically transferred to a sterile 15 ml test tube without disturbing the pellet. A bacteriophage suspension was prepared by aseptically filtering the supernatant through a 0.8 µm pore sized cellulose filter to remove particulates debris, followed by filtration through a 0.45 µm pore sized filter to remove bacterial cells and cellular debris (Beaudoin *et al*., 2007)

2.2.2 Phage Enrichment and Isolation: Escherichia coli were used as potential recipients to detect the presence of possible coliphages. Five ml of logarithmic phase-cells suspension of E coli strain were inoculated in Trypticase soy broth and mixed with (45ml) bacteriophages suspension (as prepared in above step) and10 x Trypicase soy broth (5 ml). The mixture was incubated at 37℃ with shaking at 180 rpm for 24 hr. At the end of incubation period, the suspension was clarified by centrifugation at 2500rpm for 10 minutes and the supernatant was filtered through 0.22µm pore size filters to exclude bacteria. The suspension of expected phages was kept at 4°C. (Atlas *et al*., 1995).

2.2.3 Testing the suspension of expected phages: By the spotting technique, bacteriophage lysis assay was done. An overnight culture of bacteria was spread on Trypicase soy agar and then a single drop of suspension of expected phages stock solution was added on bacterial law. The plates were inverted & incubated at 37^0C overnight, and then examined for the presence of clear zones of lysis (Douglas, 1975)

2.2.4 Purification of bacteriophages by mass multiplication: Materials from the center of the plaques were scraped off using a sterile inoculation loop and were transferred to sterile nutrient broth containing the specified organism and incubated overnight for about 18 hr. at 37^0C. This mixture was centrifuged at 5000rpm for 25min and filtered through Millipore Membrane Filter (0.22µ). The filtrate was collected in sterile amber bottles. The phage assay was again carried out as mentioned earlier. This cycle step was repeated for a minimum of thr.ee times to ensure the purity of the phages. All the phage lysated were stored at 4^0C (Sundar *et al*, 2009)

2.2.5 Lytic profiles: the lytic activities of the selected bacteriophages were determined with E coli isolates. Lytic activity was assessed by mixing 0.1 ml bacteriophage lysate containing 109- 107 PFU. The host range of phages was assessed on a range of E coli bacteria. Susceptibility of various bacterial isolates was tested using the drop-on-lawn technique (Li *et al*., 2012)

2.2.6 Titration by plaque assay: A (0.1ml) suspension of expected phages was added to (0.1 ml) of an overnight bacterial cells culture in Trypicase soy broth and then mixed with 2.5-3ml aliquots of soft agar (Trypicase soy broth containing 0.7% Bacto Agar). The mixtures were subsequently overlaid on Trypicase soy agar (Hardy diagnostics) plates prior to an overnight incubation at 37℃. A sample was indicated positive for phage when a lytic plaque type was observed on the plates. The number of phage particles was determined by plaque assay. The plaques were counted and recorded by naked eyes. Virus titers were estimated and expressed as plaque forming unit per milliliter (PFU/ml) (Pollack *et al*., 2012)

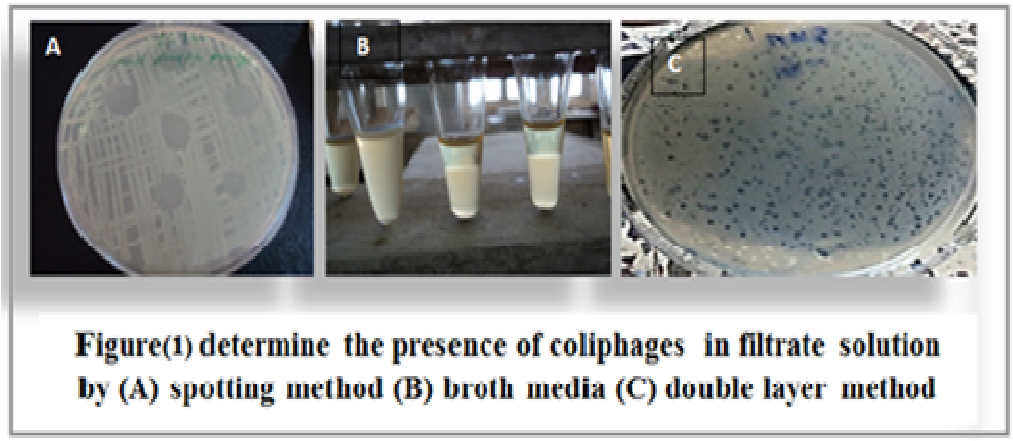
2.2.7 One-step growth curve: One-step growth curves were performed in order to determine the burst sizes and latent periods of phages at 37°C, The growth pattern of phage was determined by using a 1 ml bacterial suspension (106 CFU/ml). One ml of the virus suspension (105 PFU/ml) was added into bacterial suspension. The bacterium-phage culture was incubated with shaking at 37°C. The culture samples were harvested after 5min and then after (10, 20, 30, 40, 50 and 60min, and chloroform was added to the supernatant to kill any unlysed bacteria. Just before the culture was removed at each time interval, 0.lml of *E.coli* culture was added to a sloppy agar tube to which the culture from the water bath was added. this was then plated immediately on Nutrient agar plates and incubated overnight, the number of PFU was then determined for each time A latent period, characterized by a steady low level of viruses, a rise period characterized by a sudden increase in the number of viruses, the burst size of the virus or the number of viruses released by an individual bacterium is given by the ratio of the phage titre after the burst to that during the latent period (Benson, 2001; Atlas *et al*., 1995)

2.2.8 Stability to physical and chemical condition: Using double-layer plaque technique with phage, a (10ml) phage preparation having ($1*10^8$) PFU/ml .The heat stability of phage was tested at (-20, 4, 37 and 60 ^{0}C) in SM buffer pH 7.0(NaCl 5.8 g+ $MgSO_4.7H_2O$ 2.0 g+1M Tris-HCl (pH 7.5) 50.0 ml+2% gelatin 5.0ml+ distilled water to 1 liter) by incubation in water bath. To test the pH stability, phage was incubated overnight in SM buffer with pH varying as 2.5, 5, 7 and 9. After every 4 hr. 500 µl was aspirated from the individual vial and PFU count was performed as per the double-layer plaque techniques procedure mentioned above. This procedure was performed up to 48 hr. In addition, phage stability to sunlight tested by subjected to direct sunlight with ambient temperature ranging from 25°C to 30°C for seven days continuously. 500 µl of phage preparation was aspirated after every 24 hr., to detect PFU count as with the same protocol mentioned above (Chandra *et al*., 2011).

3. Result and Discussion

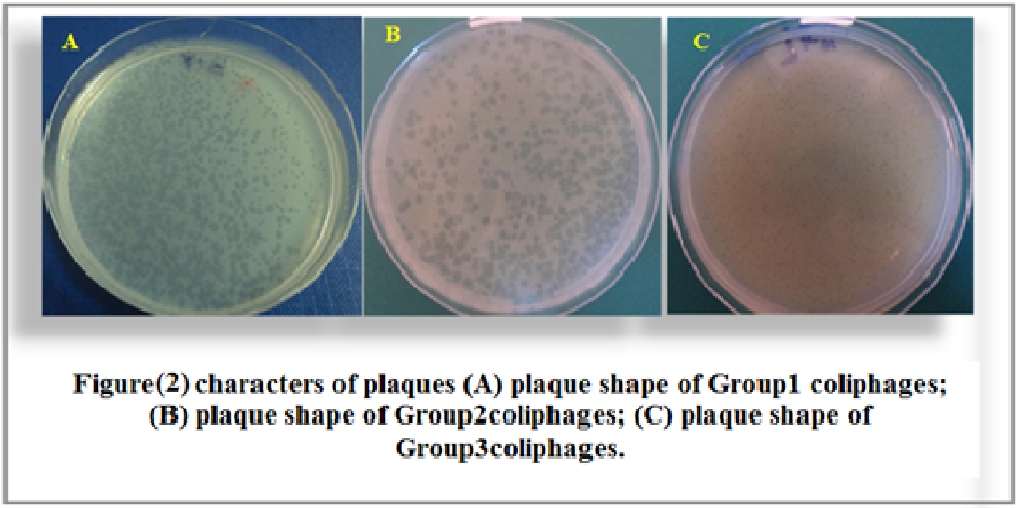
After incubation of sewage water inoculums with E coli then filtered the broth and tested for presence of

coliphage in filtered broth by spotting test, broth method or double layer method (Figure 1).



Figure(1) determine the presence of coliphages in filtrate solution by (A) spotting method (B) broth media (C) double layer method

In this study, the phage of interest is those that infect and lyses E. coli host cells. when phage are released from the ruptured host, distinct zones of clearing (plaques) form ,in order to obtain the bacteriophage, a procedure of enrichment, isolation, the presence of distinct plaques indicated that lytic bacteriophage had been successfully amplified. In this study Five phages designated (A, B, C, D and E) exhibiting potent lytic activity with clear plaques (1-4 mm in diameter) as shown in Figure (2).



Figure(2) characters of plaques (A) plaque shape of Group1 coliphages; (B) plaque shape of Group2coliphages; (C) plaque shape of Group3coliphages.

Fifty percent of the E coli strains were infected by phage isolates (Table 1) according to the phage lytic profiles, the phages were divided into 3 different groups based on the host susceptibility, as indicated on the bottom of the Table 1 each group characterized by the same lytic profile of the phages for the same host strains, which either were sensitive to the distinct phages, by showing clear or turbid plaques, or resistant (no phage plaques observed) from these results it seems very likely that the phages belonging to different groups also represent different phages.

Table (1) Lytic Profile of Coli phage

E coli \ Phage	A	B	C	D	E
1	—	—	+	+	+
2	—	—	—	—	—
3	—	—	+	+	—
4	—	—	—	—	—
5	—	—	—	—	—
6	+	+	+	+	+
7	T	T	T	T	T
8	—	—	—	—	—
9	—	—	—	—	—
10	+	+	—	—	—
11	—	—	—	—	—
12	—	—	—	—	—
13	—	—	T	T	—
14	—	—	—	—	—
15	+	+	+	+	T
16	—	—	+	+	+
17	—	—	—	—	—
18	—	—	—	—	—
19	—	—	+	+	—
20	+	+	+	+	—
21	—	—	—	—	—
22	T	T	T	T	T
23	—	—	—	—	—
24	+	+	+	+	+
25	+	+	+	+	T
26	—	—	—	—	—
27	T	T	T	T	T
28	T	T	T	T	T
29	+	+	+	+	+
30	—	—	—	—	—
31	+	+	+	+	+
32	—	—	+	+	—
33	T	T	T	T	T
34	—	—	—	—	—
35	—	—	—	—	—
36	T	T	+	+	T
37	—	—	—	—	—
38	T	T	T	T	T
39	+	+	+	+	+
40	—	—	—	—	—
41	+	+	+	+	+
42	+	+	+	+	+
43	T	T	T	T	T
44	—	—	—	—	—
45	—	—	—	—	—
46	T	T	T	T	+
47	—	—	—	—	—
48	+	+	T	T	+
49	+	+	+	+	+
50	+	+	+	+	+
Profile Group	1	1	2	2	3

*+: clear zone *—: dim zone* T: turbid zone

this study included determination of phage titer by using series of dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}) and counting the number of plaque forming units (PFU.) for each dilution it was (-,-,-,1.3×10^{6}, 8×10^{5}, 6×10^{4}, 3.7×10^{3}, 2×10^{2},0,0 respectively)this results means that the dilution gave the best countable number of plaques is (10^{-6}).

Table (2): determination of phage titer

Plate NO.	Vol. of phage plated (ml)	Serial Dilution	Dilution Factor (DF)	Plaque per plate	Titer calculation of stock= Plaque× invert Dil./ Vol.of phage plated (ml)	Result of stock Titer (PFU)	Titer calculation of dilution = Plaque× DF/ Vol.of phage plated (ml)
1	0.1	10^{-1}	10	Clear	-	-	-
2	0.1	10^{-2}	10	Clear	-	-	-
3	0.1	10^{-3}	10	Clear	-	-	-
4	0.1	10^{-4}	10	12800	$12800\times10^{4}/\ 0.1$	1.3×10^{9}	1.3×10^{6}
5	0.1	10^{-5}	10	8000	$8000\times10^{5}/\ 0.1$	8×10^{9}	8×10^{5}
6	0.1	10^{-6}	10	600	$600\times10^{6}/\ 0.1$	6×10^{9}	6×10^{4}
7	0.1	10^{-7}	10	37	$37\times10^{7}/\ 0.1$	3.7×10^{9}	3.7×10^{3}
8	0.1	10^{-8}	10	2	$2\times10^{8}/\ 0.1$	2×10^{9}	2×10^{2}
9	0.1	10^{-9}	10	0	$0\times10^{9}/\ 0.1$	0	0
10	0.1	10^{-10}	10	0	$0\times10^{10}/\ 0.1$	0	0

In this study the latent periods of phage C 20 min. Burst size for a Phage C is 160. (Calculate the burst size by dividing the concentration of bacteriophage at time=45min by the concentration of bacteriophage at time=25min) as shown in figure (3).These important characteristics vary between viruses but are often similar for related ones. Thus, they can serve as taxonomic criteria, mostly for high-level taxonomy. The number of released phages or burst size, and the time it takes to make them or latent period, are mainly determined by the complexity and size of the virion so it is expected that these criteria are conserved between phages with similar morphology (Al-Mola and Al-Yassari, 2010).

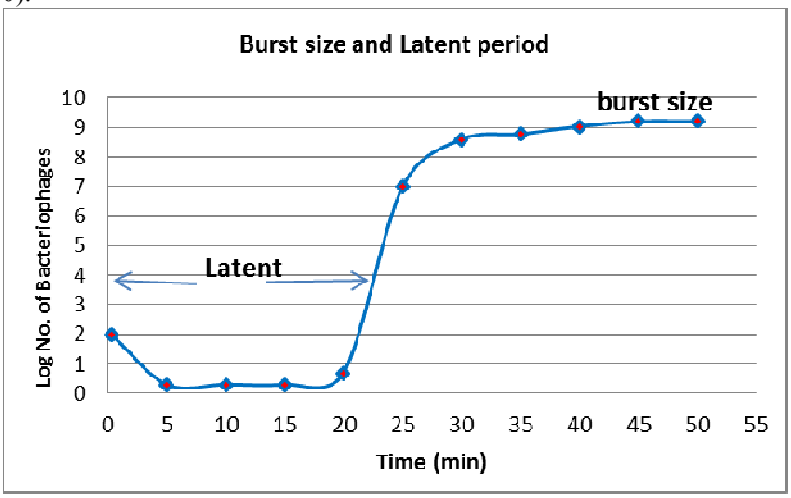


Figure (3) curve of phage growth

When this phage was subjected to different conditions of temperature (-20^{0}C-60^{0}C) For tow day with interval 6hr., Titration were done, no phage was detected after the treatment with 60^{0}C after 6hr., but return appear after 12hr.. This may be error in work. At 37^{0}C and 60^{0}C no phage was detected during 48hr., and 30hr. respectively, However phage remained infectious after treatment with Temperature -20^{0}C and 4^{0}C Table (3).

Table (3) Stability of phage C to temperature

Series	Time interval (hr.)	Phage concentration (Log 10)			
		—20C^0	4C^0	37C^0	60C^0
1	1	6.853613048	9.574031268	8.181843588	7.204119983
2	6	6	9.477121255	8.176091259	Nil
3	12	5.875061263	9.477121255	7.903089987	3.954242509
4	18	5.698970004	8.903089987	7	3.903089987
5	24	5.602059991	7.318063335	6.846955325	3.77815125
6	30	5.544068044	7.301029996	6	Nil
7	36	5.477121255	7	5.698970004	Nil
8	42	5.397940009	7	5.477121255	Nil
9	48	5.397940009	7	Nil	Nil
10	54	5.397940009	7	Nil	Nil

When this phage was subjected to various different conditions of pH (2.5 to 9), it revealed that during the first 6 hr. of pH exposure there is a definite reduction in the PFU counts but later on it was observed that the PFU count decreased (Table 4).The effect of pH when examined gave the insight that the phage can remain viable, surviving a wide variation of pH (2.5 to 9). Though, initially there was a reduction in phage count but the activity was not lost completely, indicating that the mechanism of resistance to with stand pH variation is innate with bacteriophages thus letting them survive in sewage where pH varies greatly.

Table (4) Stability of phage C to pH

Series	Time interval (hr.)	Phage concentration (Log 10)			
		pH 2.5	pH 5	pH 7	pH 9
1	1	5.484299839	6.204119983	9.204119983	7.029383778
2	6	4.301029996	6.204119983	9.204119983	7
3	12	4.176091259	6.204119983	8.204119983	7
4	18	4	3.544068044	7.204119983	5
5	24	3.477121255	5.944482672	7.204119983	4.698970004
6	30	Nil	5	6.204119983	4
7	36	Nil	4.698970004	6	3
8	42	Nil	4	5.903089987	Nil
9	48	Nil	Nil	5.84509804	Nil
10	54	Nil	Nil	5.84509804	Nil

When the effect of sun radiation was evaluated on the phages it was observed that phage count decreased when exposed directly to sunlight at almost constant ambient temperature (Table 5). The number decreased from 9.5 to 7 during the first 24 hr of exposure and thereafter the count was stabilized for next two days to diminish to 0 by the 8th day.

Table (5) Stability of phage C to sunlight

Series	Time interval (hr.)	Phage concen. (Log 10)
1	1	9.574031268
2	24	7
3	48	7.903089987
4	72	7
5	96	5
6	120	4.397940009
7	144	3.176091259
8	168	2
9	192	Nil
10	216	Nil

4. Conclusion

the isolated coliphage C is survive, fast and efficient in lysing different multidrug resistance DEC strains and

may be a good candidate to be used as a sanitation agent to control the prevalence of spoilage causing multidrug resistance DEC strains and may be as the choice of phage therapy.

5. Abbreviation

PFU = plaque forming unit
hr. = hour
DEC= diarrheagenic *Escherichia coli*

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