Original article

Competency of Coliphages Isolated from Waste Stabilization Ponds against *E. coli* strains

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Abstract

This work was carried out to study the competence of coliphages (isolated from Soba Stabilization Station, from the Anaerobic, Facultative, and Maturation ponds) against *E. coli* strains using the chloroform technique and quantified using UV-visible spectrophotometer and the double layer agar technique for plaque assay. The interaction between bacterial cultures and isolated phages was monitored using a UV-vis spectrophotometer, the optical density at OD_{600} was monitored at two stages of infection; the first infection, where it ranges between 0.405–0.686 AU, and the second infection, which gives rise to a range of optical density between 0.233–0.250 AU. In addition to the OD_{600} measurements, double layer agar technique showed variations in OD per pond and sampling site. Inhibition activities of the isolated coliphages were performed by the method of Miles and Misra (1938). The phages showed variation in inhibition activities with best result found on Luria Bertani (LB) semi solid medium.

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Introduction

Coliforms are facultative aerobic, Gram negative rods that are able to ferment lactose at 35 °C. Its presence among sewage pathogens are responsible for frequent infectious diseases in human and animals (Beheshti *et al*., 2015). Bacteriophages are viruses that are significantly distributed in nature and specifically attack bacterial hosts (Waldor*et al*., 2005; Beheshti*et al*., 2012).

Water recycling is reusing treated wastewater for commitments such as agricultural and industrial activities (Anon, 2017). The use of treated wastewater as water resources for agricultural and industrial applications is an origin for re-emergence and distribution of pathogenic bacteria (Luciana and Layara, 2017). Amongst coliforms, *Escherichia coli* is a Gram-negative rod related to order *Enterobacteriales* and family *Enterobacteriaceae*. This microorganism is the most important agent for urinary and gastrointestinal infections in human. *E. coli* is also one of the most valuable contamination index of water, food and agricultural products, indicating contamination with coliforms and wastewater (Theng Fong and Lipp, 2005).

Phages play an important role in ecology (e.g., phage impact on the cycling of organic matter in the biosphere at a global level), that phages influence the evolution of bacterial genomes (most obviously in the development of bacterial pathogenicity), and phages might provide potential tools to face the antibiotic resistance crisis in medicine. (Chennoufi *et*

al., 2004). Coliphages are useful models or surrogates to assess the behaviour of enteric viruses in water environments and the sensitivity to treatment and disinfection processes. In this regard, they are superior to faecal bacteria (Ashbolt *et al*., 2001).

Recently, bacteriophages have been used for removal of hospital wastewater pathogens such as *Pseudomonas* spp., *Streptococcus* spp., *Bacillus* spp. and antibiotic resistant *E. coli* (Periasamy and Sundaram, 2013). Bacteriophages are the most abundant microorganisms in aquatic environments and play a critical role in keeping host populations, such as algae, fungi and bacteria, under control, thus they could be used for the elimination of environmental microorganisms considered as public health threats (Collins, 2008).

Materials and methods

The standard strain of *E. coli* ATCC 25922 was kindly provided from Sudan National Centre for Research (NCR), the human Pathogenic bacteria strain of *E. coli* was obtained from Faculty of Medical Laboratory Science (Al Neelain University), this strain was isolated from urine of patient with urinary tract infection. The environmental strain was isolated from the water of the Blue Nile. This was done by adding 1 ml of the water to the surface of selective media (Eosin Methylene Blue). Colonies that appear metallic green on EMB were sub-cultured onto freshly prepared EMB plate to obtain pure isolate.

Samples collection and phage extraction

Plant Samples collections, filtration and turbidity measures were done as stated by Elshayeb *et al*., (2010). The Samples were collected from Soba stabilization station; 250 ml from the crude sewage were taken from the Anaerobic, Facultative, and Maturation ponds to isolate coliphages from the inlet, surface, bottom and outlet of each pond. Samples were immediately transported to the laboratory. The crude sewage was mixed thoroughly and filtered using Whatmann filter paper, then 5 ml were transferred to centrifuge tube and 1ml of chloroform was added. Subsequently samples were centrifuged for 15 min at 5000 rpm.1ml from the supernatant was transferred to fresh culture of *E. coli* (previously cultured by transferring single colony using loop from stock to 5 ml of nutrient broth for 24 h at 37 ºC).

Phage propagation and purification by plaque assay

This was done as reported by Rose *et al*., (1997). For this step samples were chosen as 2 samples from each pond that gave lowest absorbance, the lower absorbance indicate that the microbe has lower concentration this mean the phage has higher concentration. After incubation the bacteria were removed by centrifugation (5000 rpm for 15 min). Three ml of the supernatant were removed to another centrifuge tube, then 750 µl of chloroform were added, mixed and centrifuged again (5000 rpm for 15 min).

Phage titration and testing resistance or sensitivity

Ten-fold serial dilutions of the filtrate were made using sterile distilled water. Subsequently, 1 ml of log-phase E. coli was distributed into each of the seven tubes, labelled 10-1 – 10-7. Tubes labelled with 10-3, 10-5 and 10-7 were left at room temperature for 15 min to allow the phages to attach to the bacteria. From each dilution, 1ml was taken and added to 3ml of Tryptone Soft Agar (TSA), and immediately poured onto agar surface of base plate and left to solidify. Followed by incubation at 37ºC for 24hrs.From plates that displayed most numerous plaques, phages were obtained by adding 5ml of SM buffer (SM per liter:5.8 g NaCl, 2 g $MgSO_4.H_2O$, 50 ml Tris-HCl pH 7.5). Then plates were incubated at room temperature for 4 hrs. Consequently, the solution was taken from the plates and mixed with 1ml of chloroform, followed by centrifugation for 15min at 5000 rpm. Ten-fold serial dilutions of the supernatant were made using SM buffer. E. coli strains sensitivity to the lytic action of coliphage was tested using Miles and Misra surface drop technique (Miles and Misra, 1938;Tin-Oo *et al*., 2007; Elshayeb, 2010), 7µl

was added on the top of filter paper on the swabbed medium either Luria-Bertani (LB) semi solid medium, EMB or MacConkey, designated dilutions were 10-3, 10-5 and 10-7.

Results

Spectrophotometer reading first infection

Interactions of extracted phages and bacterial cultures turbidity measured using a spectrophotometer with wavelength 600 nm. There was reversible relationship between the increasing of the coliphages and declining of bacteria due to culture clearance which suggests the coliphages reproduction and the bacteria mortality (Table 1). The absorbency of *E. coli* bacteriophage from anaerobic pond was varied between 0.425 to 0.475 AU, maximum value exhibited in the inlet pond specimen, while minimum showed in surface specimen. In the facultative pond, the absorbency rate of the *E. coli* bacteriophage ranged between 0.405 to 0.430 AU, the maximum was revealed in surface areas while minimum presented in outlet area. Whilst In the maturation pond, the absorbency rate ranged between 0.466 AU at bottom area, whereas the surface showed absorbency rate of 0.686 AU and the inlet displayed absorbency rate of 0.489AU.

Table (1) Absorbency reading after first infection

Sample	Read1	Read2	Read3	Mean
Control –ve	0.000	0.000	0.000	0.000
Control +ve	0.479	0.475	0.475	0.476
Ai	0.474	0.478	0.472	0.475
Ab	0.457	0.463	0.462	0.461
As	0.426	0.419	0.431	0.425
Ao	0.455	0.461	0.458	0.458
Fi	0.415	0.425	0.420	0.420
Fb	0.427	0.425	0.423	0.425
Fs	0.437	0.429	0.425	0.430
Fo	0.410	0.401	0.403	0.405
Mi	0.494	0.485	0.487	0.489
Mb	0.469	0.464	0.466	0.466
Ms	0.689	0.680	0.689	0.686
Mo	0.591	0.594	0.584	0.590

Control –ve = Nutrient Broth medium, Control +ve = *E. coli* cultured medium with 1ml of sterile distilled water, Samples upper case letter (A, F and M) stand for aerobic, facultative and maturation ponds respectively. Lower case letter (i, b, s and o) stand for inlet, bottom, surface and outlet respectively.

Phage purification by plaque assay

Table (2) showing means of plaques count for the selected samples which showed lowest absorbance from each pond. Absorbance readings after second infection showed lower readings compared to readings of first infection. The readings ranged between 0.250AU to 0.233AU (Figure 1). Plaques in the plate and under microscope are shown in figure 2 and 3.

Table (2). Means of plaque titer using double layer technique

Sample	Read1	Read2	Read3	Mean
Control –Ve	0	0	0	0
Control +Ve	0.476	0.477	0.477	0.477
Plaque 1	0.253	0.250	0.247	0.250
Plaque 2	0.239	0.243	0.241	0.241
Plaque 3	0.230	0.235	0.231	0.233

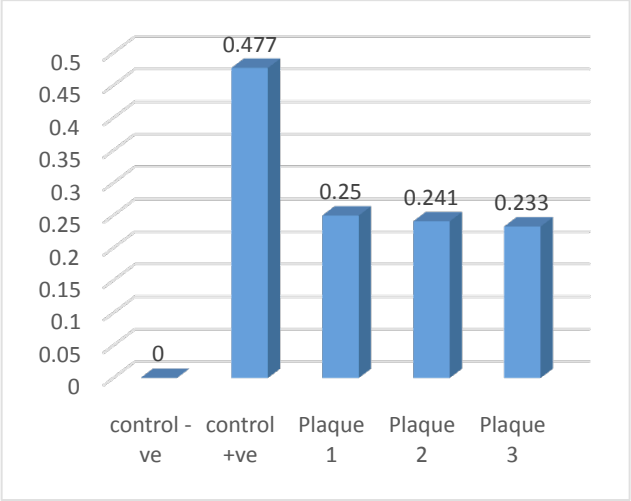

Figure 1. Means of plaque titer using double layer technique

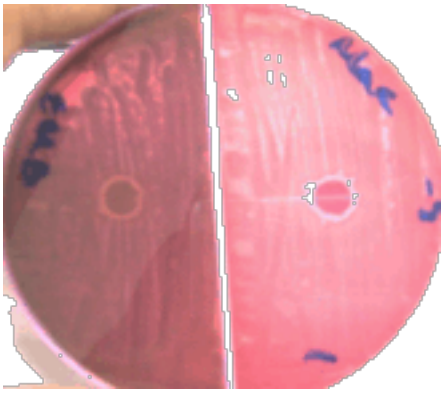

Figure 2. Miles and Misra on MacConkey medium plate (right side) and EMB (left side).

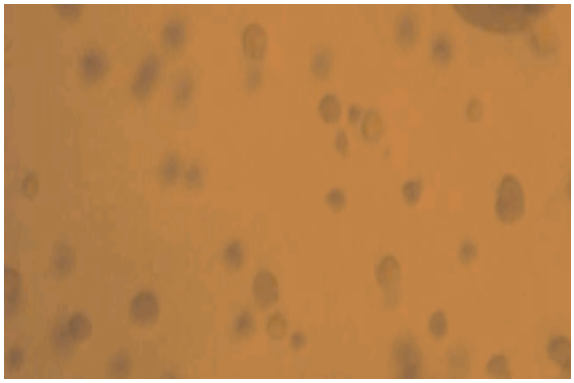

Figure 3. Plaques under microscope at 40x

Coliphages isolation using Miles and Misra surface drop technique

Results here show specification for human pathogenic *E. coli* strain in which the phage was initially isolated. The greater the phage concentration is the greater the diameter. Sample 2 was obtained from plate shows more plaque than sample 1, therefore the diameters readings are lesser for sample 1 (Table 3).

Discussion

Phages play an important role in ecology (e.g., phage impact on the cycling of organic matter in the biosphere at a global level), that phages influence the evolution of bacterial genomes (most obviously in the development of bacterial pathogenicity), and phages might provide potential tools to face the antibiotic resistance crisis in medicine. (Chennoufiet al., 2004). Coliphages are useful models or surrogates to assess the behavior of enteric viruses in water environments and the sensitivity to treatment and disinfection processes.

Selection of the host bacteria for the detection of coliphages in waste water samples is essential (Brenner et al, 1988), from the urine and water(Blue Nile water) samples *E. coli* spp. were isolated, we used a methodology previously reported by several authors (Elshayeb, 2010; Tin Oo, 2007; Rose *et al*., 199; Miles and Misra, 1938). In similar study, Elshayeb *et al*., (2011) have explained that the presence of *E.coli* phage in wastewater might be explained by natural inheritance of these organisms in the intestinal tract of both human and animals due to the natural presence of their bacterial host and these confirmed the isolation of *E. coli* phage from sewage of Soba Stabilization Station.

This study showed that there was a reversible relationship between the increasing of bacteriophages and declining of bacteria due to culture clearance. The maximum activity of extracted phages was shown in the facultative pond. For wastewater treatment in Soba Stabilization Station, the reduction in bacteria was 3.02 x 106-1.63 x 106, while the light absorbency by the *E. coli* bacteriophage was 1.0 AU (Absorbency Unit) in the anaerobic pond, 0.5 AU in the maturation pond (Elshayeb, 2011), this result doesn't support Elshayeb's conclusions in Maturation pond; this possibly due to decrease in soba stabilization station efficiency.

Maturation pond readings showed higher readings in absorbance but it didn't give plaques in double layer technique which meant the absence of coliphages from this pond except from the bottom, this high reads of absorbance in maturation pond could be explained from observation by the waste water greenish color pigmentation in compare to the other two ponds, this green color increased the intensity and thus the absorbance become higher. The greater decrease in absorbency readings in the second infection in compare to the

Table 3. Clear zone reading measured in mm.

		Luria-Bertani semi solid media						EMB			MacConkey		
		Dilution 10^{-3}		Dilution 10^{-5}		Dilution 10^{-7}		Dilution 10^{-3}	Dilution 10^{-5}	Dilution 10^{-7}	Dilution 10^{-3}	Dilution 10^{-5}	Dilution 10^{-7}
Pathogenic *E. coli*	sample 1	20	19	18	15	0	0	9	10	0	12	11	0
	sample 2	25	27	19	18	13	12	9	6	8	12	10	9
Environmntal *E. coli*	sample 1	9	9	0	0	0	0	0	0	0	0	0	0
	sample 2	10	11	0	0	0	0	0	0	0	0	0	0
E. coli ATCC 25922	sample 1	0	0	0	0	0	0	ND	ND	ND	ND	ND	ND
	sample 2	0	0	0	0	0	0	ND	ND	ND	ND	ND	ND

ND= Not Detected

readings in the first infection; it is due to phage multiplication and the greater drop in bacterial cell number, and this possibly a reversible relationship between the increasing of bacteriophages and declining of bacteria due to culture clearance (Smith, 2008).

In this study, the plaque forming unites rang between 4.60E+06 - 1.27E+09, this large population is due to the large quantity of its host in the normal environment (sewage water), and because of initial propagation in the first infection before applying double layer technique. Lytic phages causing bacterial cell lyses and this achieved by an enzyme called endolysin, which attacks and breaks down the cell wall peptidoglycan, as with the titration by double layer technique, the number of plaques must fall between 30 and 300 (Rose *et al*., 1997).The isolated coliphages showed broad lytic spectra and could lyses not only the environmental isolates of *E. coli* and above the human pathogenic *E. coli* strain Phage phiCcoIBB12, phiCcoIBB35 and phiCcoIBB37 showed broad lytic spectra and could lyses not only the *Campylobacter coli* strains but also the clinical isolates of *C. jejuni* of different flaA types. These phages are considered good candidates for phage therapy (Carvalho *et al*., 2010).

Isolated phages showed higher activity against human pathogenic *E. coli* strain compared to the two other strains. Phages are specific to their hosts and it is adsorbing to specific receptor sites on the bacterial cell wall (Ganguly *et al*., 2002). The motility of the *E. coli* phages gives a positive linear equation; meanwhile the negative equation indicates the non-motile *S. aureus* phages (Elshayeb, 2010), this finding could explain the variation of inhibition zone for the same sample.

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