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Coliphages Lysing *Escherichia coli* Bacteria: Their Morphology and Their Response to Some Physical Factors

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Abstract: Three coliphages designated ECP_1, ECP_2 and ECP_3 were identified in this study. Also, the effect of some physical factors on the infectivity of these coliphages was studied. The three coliphages were tested for their infectivity using the indicator bacterial strains *E. coli* W_1 and *E. coli* W_2 where coliphage ECP_1 was highly specific to infect *E. coli* W_1, on the other hand, coliphages ECP_2 and ECP_3 were highly specific to infect *E. coli* W_2. The three coliphages were very sensitive to heat inactivation no survivors could be recorded after 10 min of exposure time up to 60°C. Also, the profile of the three coliphages inactivation after different time intervals exposed to UV-irradiation at different heights of 15 and 30 cm from the irradiation source was examined. Increased UV-irradiation doses decreased the plaque numbers of the three coliphages tested. The effect of different pH values on stability of the three coliphages was also tested. Maximum stability was observed at pH 6.0. The viabilities of the three coliphages were rapidly decreased towards alkalinity compared to pH 6.0. The ability of the three coliphages to form plaques at preservative temperature range 3-5, 25-28 and 35-37°C was examined. The suitable storage temperatures for the three coliphages were 3-5 and 25-28°C.

Key words: *E. coli*, coliphages, sewage water, raw water, temperature, UV-irradiation, pH value, storage

INTRODUCTION

Bacteriophages (phages) are viruses which infect bacteria. Coliphages are phages that infect *E. coli* bacteria. Lytic phages can provide a natural and nontoxic method for detecting and controlling the growth of human pathogens (Ackermann and DuBow, 1987; Enan *et al*., 2012b; Abdallah *et al*., 2013). Coliphages infecting *E. coli* bacteria were used in many previous studies as biological indicators of fecal contamination of water (Grabow *et al*., 1998). Coliphages are taxonomically very diverse, covering six virus families viz. Myoviridae, Styloviriae, Podoviridae, Microviridae, Inoviridae and Leviviridae (Colford *et al*., 2007).

The use of coliphages as biological indicators of microbial contamination faces some limitations concerning difficulty of detection methods using agar plates in analyzing larger volumes of water. In addition coliphages or other phages did not denote on all bacterial types of water (Love and Sobsey, 2007). Consequently, it is better to use phages to control pathogens in water such as the use of coliphage to control growth of *E. coli* in water (Levy *et al*., 1988). It is also recommended to use phages as biotherapy (Sulakvelidze *et al*., 2001). Search on safe biotherapy is of interest and there is a need to concentrate scientific research on this point, because previous researchers recommended the use of phage in control of some human pathogens (Chanishvili *et al*., 2001) and also in control of veterinary pathogens (Matsuzaki *et al*., 2003; Enan *et al*., 2012b; Abdallah *et al*., 2013). The present research was undertaken to characterize three coliphages infecting *E. coli* W_1 and *E. coli* W_2 bacteria all were isolated from water. This is to start research about control of *E. coli* strains in water by their coliphages.

MATERIALS AND METHODS

Isolation of bacterial strains and coliphages: HiCrom *E. coli* selective agar media (HiCrom™) were used for isolation of the experimental *E. coli* W_1 and *E. coli* W_2 strains. Two *E. coli* strains were isolated from polluted raw water that was collected from the River Nile in Eastern part of Egypt. Those two strains were designated *E. coli* W_1 and *E. coli* W_2 and were used as an indicator bacterial strains for isolation and propagation of coliphages.

Coliphages infecting *E. coli* W_1 and *E. coli* W_2 strains were isolated from sewage water samples of sewage plant of Bahr El bakar canal in Western part of Egypt. Sewage water samples were filtered through membrane filters (0.45μm Millipore, Amicon) to get water

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free bacteria. The filtrate was inoculated with the indicator *E. coli* W_1 and *E. coli* W_2 strains and was further inoculated with an equal volume of nutrient broth (Oxoid) for 24 h. This mixture was centrifuged at 3000 rpm for 30 min. After removing precipitates, the mixture was passed through membrane filter (0.45 μm Millipore, Amicon) to remove heavy metals of high molecular mass. The filtered supernatants were then filtered and assayed for phage activity by double layer agar technique using soft nutrient agar (Adams, 1959; Enan *et al*., 2012b; Abdallah *et al*., 2013). Plaques of coliphages were picked up and propagated in plates to give confluent lysis. To attain coliphages with high titers, plates developed obvious plaques were washed and their fluid of washes were treated with chloroform at final concentrations 0.5% (Jensen *et al*., 1998; Enan *et al*., 2012b; Abdallah *et al*., 2013).

Identification of indicator bacteria: The criteria reported by Enan *et al.* (2013) were used for identification of bacteria at the genus level. Consequently, Gram staining, cell morphology, motility, catalase reaction, oxidase test, production of H_2S and growth on HiCrom media confirmed that the W_1 and W_2 strains belong to genus *Escherichia*. The species was determined via API 2O kits as reported by the manufacturer's instructions (Biomereux, France). Identification was carried out according to Garrity *et al.* (2005) and Enan *et al.* (2012a, 2013).

Electron microscopy study: Specimens were prepared for electron microscopic examination from partially purified coliphage or 50 mL filtered coliphage suspension (from confluent lysis plates) which was precipitated at 20,000 rpm of ice cooling centrifuge at 4°C for 24 h (Adams, 1959). Drops of high titer filtered coliphage suspension were laid on formavar coated copper grid (400 meshes) with carbon coated colloidion membrane and then negatively stained by uranyl acetate (4% aqueous). Excess fluids were withdrawn by a filter paper strep (Monod *et al*., 1997). Specimens were washed 3 times by distilled water, dried by filter paper or air dried and viewed by electron microscopy. Morphological assessment of the isolated coliphages was preformed by transmission electron microscopy (JEOL JEM.1010) at 80 kV and magnification range of 50X-500KX (Electron microscopy unit, Faculty of Medicine, Zagazig University, Egypt).

Effect of temperature on the infectivity of the three coliphages ECP_1, ECP_2 and ECP_3: The 2 mL of each coliphage suspension were incubated singly at 30, 35, 40, 45, 50, 55 and 60°C for 10 min (Dhar and Ramkrishna, 1987). Phage infectivity was determined by plaque assay technique (Adams, 1959).

Effect of UV-irradiation on the infectivity of the three coliphages ECP_1, ECP_2 and ECP_3: Sensitivity to UV-irradiation was determined by exposing 10 mL of coliphage suspension, separately, in a petri dish directly to UV light at distances of 15 or 30 cm from the UV source (240 nm). A 30 W general electric lamp was used as UV source. All manipulation was made in the dark to avoid photoreaction. Samples of phage suspensions were exposed separately to UV-irradiation for different time periods (30, 60, 90 to 180 min) and the samples were assayed by double layer technique (Dhar and Ramkrishna, 1987).

Effect of pH on the infectivity of the three coliphages ECP_1, ECP_2 and ECP_3: Coliphage suspensions were incubated at 37°C for 30 min in nutrient broth at pH values ranging from 4-10 and the survived coliphage was determined by double layer technique by using *E. coli* W_1 and *E. coli* W_2 bacteria as an indicator strains.

Effect of different storage temperatures on the coliphages ECP_1, ECP_2 and ECP_3 infectivity: Coliphage suspensions of high titer lysates were incubated in 3-5, 25-28 and 35-37°C separately for 5 weeks. The infectivity of coliphages was assayed weekly by double layer technique as described above (Dhar and Ramkrishna, 1987; Abdallah *et al*., 2013).

RESULTS

Isolation and identification of bacteria: Representatives of colonies growing on HiCrom media (HiCrom™) were picked up and purified on the same media. All the bacterial isolates were rod shaped, motile, Gram negative and catalase positive cells. Based on the manufacturer's instructions of API 2O Streps, all bacterial isolates showed positive results with regard to indole test, methyl red test, utilization of glucose, lactose, maltose, mannitole, L-arabinose, D-sorbitol but showed negative results Regarding Coagulase test, Oxidase test, Voges Proskauer test, citrate utilization, H_2S production and utilization of sucrose and salicin.

Following diagnostic key of Garrity *et al*. (2005), all the Escherichia isolates were identified and classified as belonging to *E. coli*. Two strains were selected according to their sensitivity to coliphages and designated *E. coli* W_1 and *E. coli* W_2.

Isolation and purification of coliphages: Three coliphages were isolated from sewage water using the spot test and double layer method of plaques assay (Fig. 1). Based on plaques appearance, three coliphages were chosen. Coliphage$_1$ (ECP_1) showed small clear circular plaques but

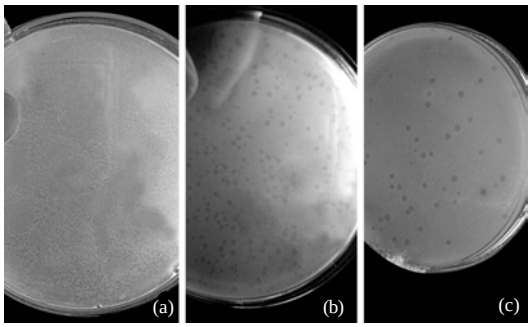

Fig. 1: Plaques morphology of the isolated coliphages after 24 h of incubation; a) plaques of coliphage ECP_1 showing small clear circular plaques; b) plaques of coliphage ECP_2 showing moderate clear circular plaques and c) plaques of coliphage ECP_3 showing large clear circular plaques

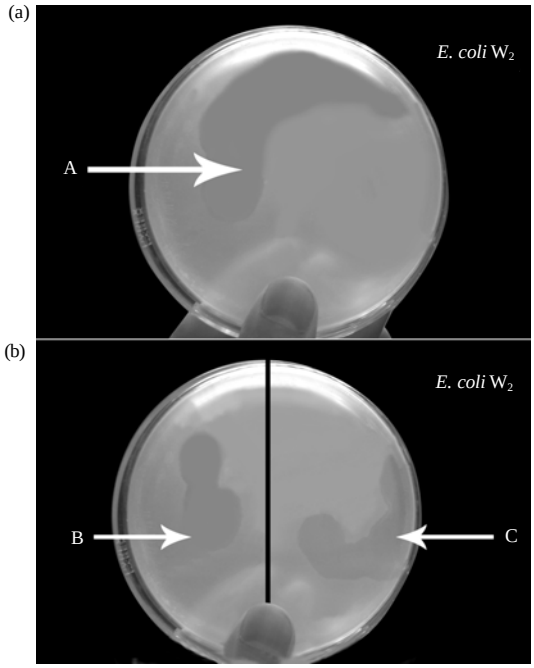

Fig. 2: a, b) Effect of spotting of the isolated coliphages on the isolated bacterial strains (*E. coli* W_1 and *E. coli* W_2); A) ECP_1; B) ECP_2 and C) ECP_3

coliphage$_2$, coliphage$_3$ (ECP_2, ECP_3) showed moderate, large clear circular plaques respectively (Fig. 1). ECP_1, ECP_2, ECP_3 were propagated and purified for further study. A large clear zones have been developed after spotting of purified coliphage on lawn of bacterial indicator by 14 h (Fig. 2).

Morphology of coliphages ECP_1, ECP_2 and ECP_3: Transmission electron microscopy was employed to observe the morphology of coliphages ECP_1, ECP_2 and ECP_3 (Fig. 3). The coliphages ECP_1 and ECP_2 had isometric hexagonal head (50×40 and 90×70 nm, respectively) while coliphage ECP_3 possessed elongated hexagonal head (120×100 nm). The three coliphages had contractile tails and their lengths were 40×10, 80×10 and 200×40 nm for ECP_1, ECP_2 and ECP_3, respectively (Fig. 3). Consequently, the coliphages ECP_1, ECP_2 and ECP_3 were classified as belonging to family Myoviridae (group A) (Bradley, 1967; Mathews, 1982).

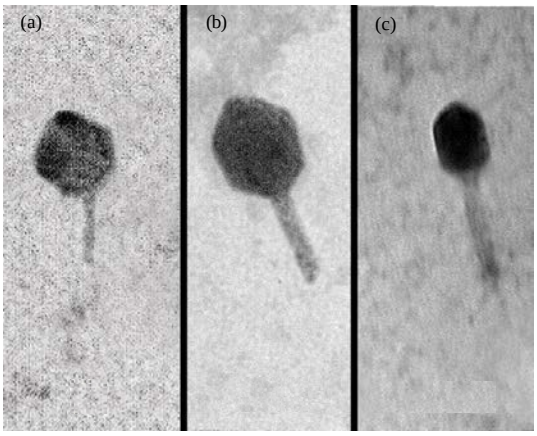

Fig. 3: Electron microscopy of the isolated coliphages; a) ECP_1; b) ECP_2 and c) ECP_3

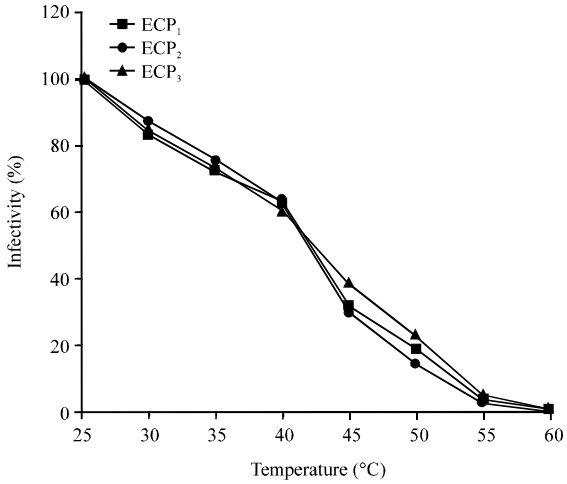

Fig. 4: Effect of temperature on the infectivity of the three coliphages

Effect of temperature on the infectivity of the three coliphages ECP_1, ECP_2 and ECP_3: Coliphages ECP_1, ECP_2 and ECP_3 were highly sensitive to heat inactivation (Fig. 4). No survivors could be recorded after exposure of these coliphages to 60°C. The highest numbers of plaques were observed at 30°C, reaching 83.2, 87.2 and 84.1% for

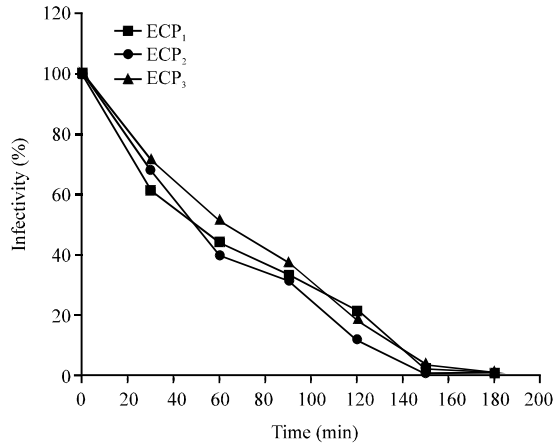

Fig. 5: Inhibition of the three coliphages by exposing to UV-irradiation (at a distance of 15 cm from the UV source)

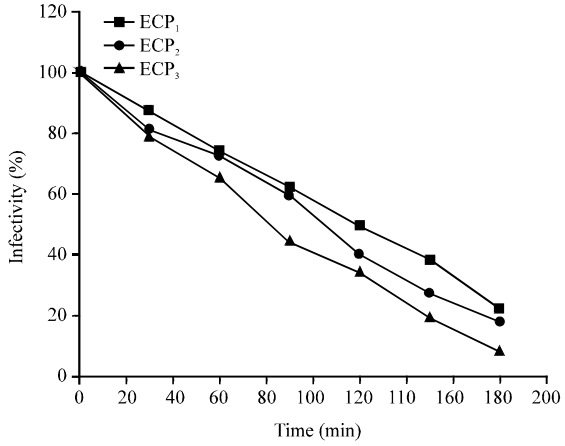

Fig. 6: Inhibition of the three coliphages by exposing to UV-irradiation (at a distance of 30 cm from the UV source)

the three coliphages, respectively. The viability of the three coliphages was decreased with increasing the temperature.

Effect of UV-irradiation on the infectivity of the three experimental coliphages: The exposure of coliphages to the UV-irradiation had inhibitory effect on the coliphage infectivity and the increasing of the exposure time caused decreasing in the coliphage infectivity (Fig. 5 and 6).

At 15 cm height from UV source, a 180 min exposure was sufficient for complete inactivation of coliphages. In addition, ECP_2 was inactivated completely at 150 min. Therefore, ECP_2 was the most sensitive to UV-irradiation than ECP_1 and ECP_3.

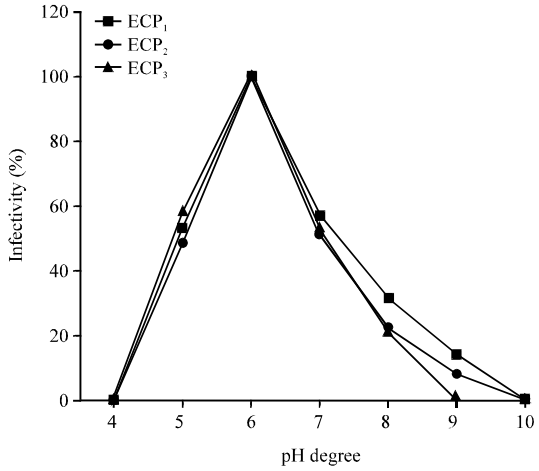

Fig. 7: Effect of pH value on the infectivity of the three coliphages

At 30 cm height from UV source, a 180 min exposure was not sufficient for complete inactivation of coliphages ECP_1, ECP_2 and ECP_3. A 77.9, 81.9 and 91.6% inactivation were observed after 180 min of exposure to UV-irradiation for coliphages ECP_1, ECP_2 and ECP_3, respectively. Surprisingly no change in plaque morphology was observed after UV-irradiation under the studied conditions.

Effect of pH on the infectivity of the three coliphages ECP_1, ECP_2 and ECP_3: The results shown in Fig. 7 showed that the optimum pH values for the three coliphages were determined by incubation of coliphage suspensions at different pH values ranging from 4-10 for 30 min. Then the remaining coliphages particles were survived by double layer technique. As observed in Fig. 7, the three coliphages were very sensitive to hydrogen ion concentrations. The optimum pH 6.0 was the best pH value for the coliphages stability. The viability of the three coliphages was rapidly decreased towards alkalinity compared to pH 6.0. Moreover, the plaques formation of the coliphage ECP_3 was more sensitive to hydrogen ions exchange than coliphages ECP_1 and ECP_2

Effect of different storage temperatures on the coliphages ECP_1, ECP_2 and ECP_3 infectivity: The ability of ECP_1, ECP_2 and ECP_3 to form plaques at different storage temperatures was determined using *E. coli* W_1 and *E. coli* W_2 as an indicator strains by double layer technique. The 3-5 and 25-28°C temperatures were suitable for coliphage infectivity while the 35-37°C temperature was suitable for coliphage infectivity till the 5th week for ECP_3

Table 1: Effect of different storage temperatures on coliphages (ECP_1, ECP_2 and ECP_3) infectivity

		ECP_1		ECP_2		ECP_3	
Temperature	Time (weeks)	PFU/mL $\times 10^7$	Percentage	PFU/mL $\times 10^7$	Percentage	PFU/mL $\times 10^7$	Percentage
Control	0	2.99	100.0	4.06	100.0	4.93	100.0
3-5°C	1	2.78	93.2	3.88	95.6	4.78	97.1
	2	2.45	82.1	3.54	87.4	4.50	91.4
	3	2.23	74.6	2.96	73.1	4.05	82.3
	4	1.82	61.2	2.63	64.8	3.67	74.6
	5	1.44	48.4	2.12	52.3	3.25	66.1
25-28°C	1	2.66	89.1	3.72	91.7	4.61	93.7
	2	2.29	76.8	3.22	79.5	4.19	85.1
	3	1.77	59.2	2.37	58.4	3.26	66.2
	4	1.31	44.1	1.63	40.2	2.67	54.3
	5	0.78	26.1	1.26	31.1	2.11	42.9
35-37°C	1	2.52	84.3	3.55	87.6	4.50	91.4
	2	1.98	66.5	2.63	64.9	3.76	76.4
	3	1.17	39.2	1.37	33.8	2.81	57.1
	4	0.49	16.4	0.53	13.2	1.62	32.9
	5	0.00	0.0	0.00	0.0	0.97	19.7

and the 4th week to ECP_1 and ECP_2. So, ECP_1 and ECP_2 were more sensitive to the 35-37°C temperature than ECP_3 as shown in Table 1.

DISCUSSION

The growth of bacterial isolates on HiCrom medium and other biochemical, morphological and cultural criteria facilitated the identification of *E. coli* strains isolated from water (Garrity *et al*., 2005; Enan *et al*., 2012a, 2013). Three coliphages named ECP_1, ECP_2 and ECP_3 were isolated from sewage water. ECP_1 was specific for *E. coli* W_1 while the other ECP_2 and ECP_3 were specific for *E. coli* W_2. According to the morphological features appeared by electron microscopy and based on taxonomic criteria reported by Bradley (1967) and Mathews (1982), the three coliphages ECP_1, ECP_2 and ECP_3 were classified as belonging to group A of family Myoviridae.

Thermal inactivation pattern showed that the three coliphages ECP_1, ECP_2 and ECP_3 were highly sensitive to elevated temperature and the numbers of plaque forming units were greatly reduced. The obtained results revealed that the three coliphages were inactivated after exposure to 60°C for 10 min. These results were confirmed by Kuroda and Bradley (1968) who mentioned that three phages named SAP1, SAP2 and SAP3 were inactivated by exposure to 60°C for 1 h. They found that the replication of these phages were least affected by 40°C. El-Tarabily *et al*. (1995) found that the phage numbers ΦS1, ΦS2 and ΦS3 specified to *Streptomyces diastaticus*, *Streptomyces griseus* and *Streptomyces hygroscopicus*, respectively were markedly decreased by exposing to 45 or 56°C for 15 min. The infectivity of the three coliphages gradually decreased with increasing the time and decreasing distance of exposure to UV-irradiation till the complete inhibition taken place. The three coliphages were completely inhibited after exposure to this irradiation at a distance of 15 cm for 150 min (coliphage ECP_2) or 180 min (coliphages ECP_1 and ECP_3). On the contrast the coliphages were still infective after exposure to UV-irradiation at 30 cm for 180 min. Therefore, these coliphages were resistant to exposure to UV-irradiation and similar results were published by Dhar and Ramkrishna (1987) and El-Helali (2001).

Stability of the three coliphages of current study at different pH values was investigated. The three coliphages were very sensitive to change in the hydrogen ions concentration with an optimum value of pH 6.0. The viability of these coliphages was rapidly decreased towards alkalinity. At pH 10.0, plaques formation were not observed for ECP_1 and ECP_2 while at pH 9.0, plaques formation were not observed for ECP_3. Therefore, the respective percentages of survivors at pH 5.0 were 53% for ECP_1, 48% for ECP_2 and 58% for ECP_3 compared to counts at pH 6.0. At pH 7.0, 8.0 or 9.0, the respective percentage of survivors reached 56, 31 and 14% for ECP_1 or 51, 22 and 8% for ECP_2 or 53, 20 and 0% for ECP_3, respectively compared to counts at pH 6.0. Delbruck (1950) revealed that pH value of the suspending medium was an important factor affecting stability of phages and similar results in this respect were published by Jang and Kim (1996). Sykes *et al*. (1981) found that a wide range (5.5-9.0) of pH values were valuable for the stability of phages virulent on acidophilic Streptomyces. The optimum pH value for the *Streptomyces griseus* phages were generally between 7.0 and 8.0 these values have, therefore been used for the satisfactory storage of the phage for at least 2 years (Change, 1953).

On the other hand, Rautenshtein *et al*. (1975) reported that the particles of temperate phage infecting *Streptomyces rimosus*, a producer of oxytetrecyclin, lost lytic activity by incubation at pH 4.0 for 15 min.

CONCLUSION

The viability of the three coliphages ECP_1, ECP_2 and ECP_3 in different storage temperatures were found to be more stable at 3-5 or 25-28°C than 35-37°C. The infectivity of the three coliphages was lost with prolonged incubation time. Similar results were published in this respect (El-Tarabily *et al*., 1995; Patten *et al*., 1995). Further research will be necessary regarding complete physicochemical and biological characters of the coliphages employed herein. This will be important to start research about use of the coliphages ECP_1, ECP_2 and ECP_3 for inhibition of *E. coli in vitro* and in water.

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