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Evaluation of the use of Novel Coliphages to Control *Escherichia coli* W_1 and *Escherichia coli* W_2 Strains Isolated from Water

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Abstract: This research described a preliminary studies for controlling two *Escherichia coli* strains (*E. coli* W_1 and *E. coli* W_2) isolated from polluted water by their novel coliphages which identified herein and designated ECP_1, ECP_2 and ECP_3. *E. coli* W_1 and *E. coli* W_2 strains were isolated from polluted water and identified using usual cultural, Morphological and Biochemical Methods. Three bacteriophages lysing *E. coli* strains were isolated. The bacteriophage lysing *E. coli* W_1 (coliphage$_1$) was designated ECP_1 while coliphage$_2$, coliphage$_3$ were highly specific to infect *E. coli* W_2 and consequently have been designated ECP_2, ECP_3, respectively. Transmission electron microscopy showed that the ECP_1, ECP_2 and ECP_3 coliphages belong to family Myoviridae. Increasing host age lead to inhibition of coliphage infectivity. The ECP_1, ECP_2 and ECP_3 coliphages exhibited different rates of adsorption and burst sizes but they possessed the same latent and rise periods (30 min). The host range of the three coliphages suggested that these coliphages may be useful as biocontrol agents. Addition of ECP_1, ECP_2 and ECP_3 coliphages to their hosts decreased viable cell population of their host *E. coli* W_1, W_2 distinctively *in vitro*.

Key words: *Escherichia coli* W_1 and *Escherichia coli* W_2 (*E. coli* W_1 and *E. coli* W_2), *E. coli* phages$_{1-3}$ (ECP_{1-3}), electron microscopy, adsorption, biocontrol

INTRODUCTION

Bacteriophages (phages) are viruses that infect bacteria. Like all viruses, phages are obligate intracellular parasites which have no intrinsic metabolism and require the metabolic machinery of the host cell to support their reproduction. Bacteriophages are highly abundant in the aquatic environment ranging from 10^4 PFU mL^{-1} to in excess of 10^8 PFU mL^{-1} (Bergh *et al*., 1989). Coliphages are bacteriophages that infect *E. coli* bacteria. Lytic phages can provide a natural and nontoxic method for detecting and controlling the growth of human pathogens. Phages are parts of both gastrointestinal and environmental ecosystems (Ackermann and DuBow, 1987; Enan *et al*., 2012a; Abdallah *et al*., 2013).

Historically, phages have been employed as therapeutic agents in diagnostic tests to detect bacterial pathogens and as biological control agents to reduce and eliminate food borne pathogens in food (Modi *et al*., 2001). Bacteriophages have bactericidal properties, so can be used in the control of clinical bacteria. Phages were used widely in the early 20th century to treat human and animal illness with varying degrees of success. Phage therapy research in Eastern Europe shows that phage treatments against a wide array of bacteria including staphylococci, pseudomonads, *Proteus* sp. and enteric pathogens were produced (Chanishvili *et al*., 2001). Phages have been employed as biocontrol agents capable of eliminating or reducing the levels of bacterial pathogens in or on food by taking advantage of the ability of virulent phages to lyse and thereby kill, the target organism (Modi *et al*., 2001).

First described by Escherich in 1885 (Escherich and Bettelheim, 1989), *E. coli* is the most abundant facultative anaerobe of the normal intestinal flora and is responsible for producing what is perceived as the widest spectrum of disease of any bacterial species (Keusch and Thea, 1993). This study described the biological and morphological characterization of three coliphages viz. ECP_1, ECP_2 and ECP_3 which infected *E. coli* W_1, W_2 strains. In addition a preliminary studies for controlling *E. coli* W_1, W_2 strains isolated from polluted water were described.

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, W_2 strains. Two

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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, 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 (0.45 μm Millipore, Amicon) to get water free bacteria. The filtrate was inoculated with the indicator *E. coli* W_1, W_2 strains and 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*., 2012a; 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*., 2012a; 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, b, 2013).

Electron microscopy study: Specimens were prepared for electron microscopic examination from partially purified coliphage or 50 mL filtered of each 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 the host age on the infectivity of the three coliphages ECP_{1-3}: Cultures of indicator bacterial strains (*E. coli* W_1 and *E. coli* W_2) at different ages (24, 48, 72 and 96 h) were prepared. The titer (PFU/mL) of each coliphage at each age of its host was determined by plaque assay technique.

Determination of host range: The isolated coliphage stocks were obtained by their propagation on the isolated bacterial strains (*E. coli* W_1 and *E. coli* W_2). Overnight cultures of different strains of different bacterial species listed in Table 1 were used in seeding double layer agar plates. Such plates were spotted with a drop of each isolated coliphage enriched suspension. Plates were examined for lysis after 24 h of incubation at 37°C (Schnabel and Jones, 2001).

Adsorption experiment: Each filtered lysate of the tested coliphage ECP_1 was added to a log phase of *E. coli* W_1 and ECP_2 and ECP_3 were added to log phase cells of *E. coli* W_2, respectively as an indicator strain at multiplicity of infection = 100 PFU/CFU/mL (Stent, 1963). The 1 mL samples were withdrawn and filtered through Millipore membrane filter (0.45 μm) after 4 min intervals and assayed by the double layer technique to determine the titer of unadsorbed coliphages. Adsorption rate constant was determined by the following equation (Adams, 1959):

Table 1: Host range of the isolated coliphages

		Lytic areas formation		
Isolates	Source	ECP_1	ECP_2	ECP_3
E. coli W_1	Present study	+	-	-
E. coli W_2	Present study	-	+	+
E. coli W_3	Present study	-	-	-
E. coli W_4	Present study	-	-	-
E. coli W_5	Present study	-	-	-
E. coli ATCC 8739	Reference Lab.[a]	-	-	-
E. coli NCTC 12241	Reference Lab.[a]	-	-	-
Staphylococcus aureus NCTC 12981	Reference Lab.[a]	-	-	-
S. aureus 6538	Reference Lab.[a]	-	-	-
Klebsiella pneumonia	Reference Lab.[a]	-	-	-
Enterobacter aerogenes 13048	Reference Lab.[a]	-	-	-
Salmonella typhimurium 14028	Reference Lab.[a]	-	-	-
Pseudomonas aeruginosa 9027	Reference Lab.[a]	-	-	-
Aeromonas hydrophila	Reference Lab.[a]	-	-	-

'+': Presence of lytic areas; '-': Absence of lytic areas; a: Reference laboratory for potable water, holding company for potable water and sanitation, Cairo, Egypt

$$K = (2.3/BT).\ Log\ (P_0/P)$$

Where:

K = Adsorption rate constant
B = Bacterial host concentration
T = Time
P_0 = Unadsorbed coliphage concentration at the beginning
P = Unadsorbed coliphage concentration at the end

One-step growth curve experiment: Indicator bacterial strains: *E. coli* W_1 and *E. coli* W_2 were infected by coliphages at Multiplicity of Infection (MOI) of 0.1 PFU/CFU/mL and incubated at 37°C. After 40 min, each mixture was centrifuged at 3000 rpm for 10 min. The pellet was re-suspended in 2 mL nutrient broth followed by dilution and counting the bacteria. After 10 min intervals, 0.1 mL samples were withdrawn and plated onto soft agar with the indicator bacterial strains *E. coli* W_1 and *E. coli* W_2. The observed numbers of coliphages in suspensions were plotted against time and the latent period, rise period and burst sizes were determined. (Adams, 1959).

Biocontrol of *E. coli* W_1 and *E. coli* W_2 strains by their specific coliphages: Nutrient broth (Oxoid) was prepared and inoculated by either *E. coli* W_1 or *E. coli* W_2 strains at final concentrations of about 2.1×10^7 CFU mL^{-1}. ECP_1, ECP_2 or ECP_3 at titers of about 0.01×10^6 (10^4) PFU mL^{-1} were prepared. The ECP_1 suspension was added to its specific *E. coli* W_1 cells and either ECP_2 or ECP_3 was added to its specific *E. coli* W_2 cell suspension. Every 12 h, 1 mL samples were removed and viable cell populations (CFU/mL) were determined (Enan *et al*., 2013). In addition, titers of coliphages (PFU/mL) were also determined by double layer technique (Adams, 1959; 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 $coliphages_{2, 3}$ (ECP_2, ECP_3) showed moderate, large clear circular plaques, respectively (Fig. 1). ECP_{1-3} 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 nm; 80×10 and 200×40 nm for ECP_1; ECP_2; 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).

Effect of the host age on the infectivity of the three coliphages ECP_{1-3}: Data in Fig. 4 indicated that the infectivity of the three coliphages ECP_1, ECP_2 and ECP_3 decreased with the increase of the host age. The infectivity of ECP_1 and ECP_3 coliphages were more sensitive to the age of the host; their infectivity reached 25.2 and 23.6%, respectively after 96 h of host age. On the

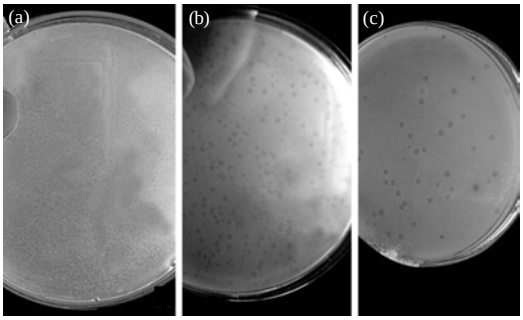

Fig. 1: Plaques morphology of the isolated coliphages after 24 h of incubation; a) plaques of coliphage ECP_1 showing small clear circular plaques; b) plaques of coliphage ECP_2 showing moderate clear circular plaques and c) plaques of coliphage ECP_3 showing large clear circular plaques

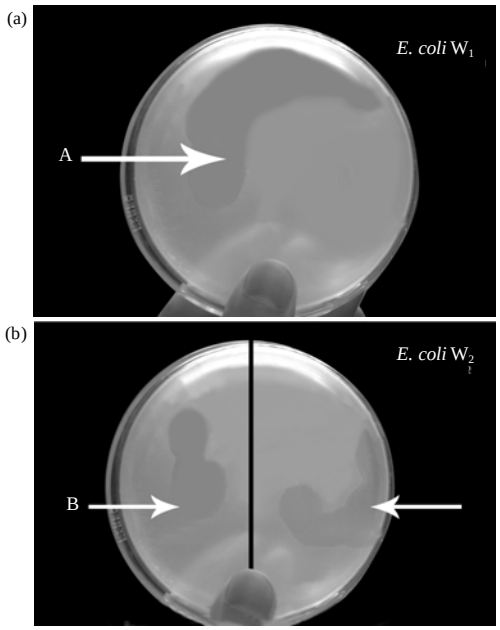

Fig. 2: a, b) Effect of spotting of the isolated coliphages on the isolated bacterial strains (*E. coli* W_1 and *E. coli* W_2); A) ECP_1; B) ECP_2 and C) ECP_3

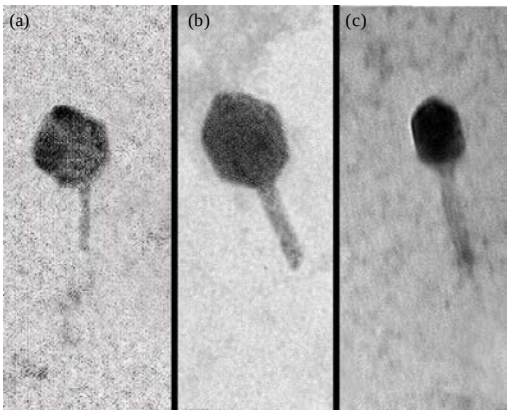

Fig. 3: Electron microscopy of the isolated coliphages; a) ECP_1; b) ECP_2 and c) ECP_3

contrast, coliphage ECP_2 was the most resistant to host age where its infectivity reached 58.8% after 96 h of incubation.

Host range: The three coliphages ECP_{1-3} were assayed against 7 strains of *Escherichia coli* in addition to 7 various bacterial species including 2 strains of *Staphylococcus aureus* in addition to one strain of the 5 following species: *Klebsiella pneumonia*, *Enterobacter aerogenes* 13048, *Salmonella typhimurium* 14028, *Pseudomonas aeruginosa* 9027 and *Aeromonas*

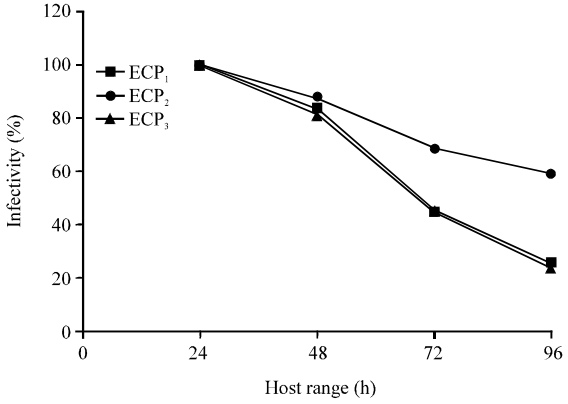

Fig. 4: Effect of the host age on the infectivity of the isolated coliphages

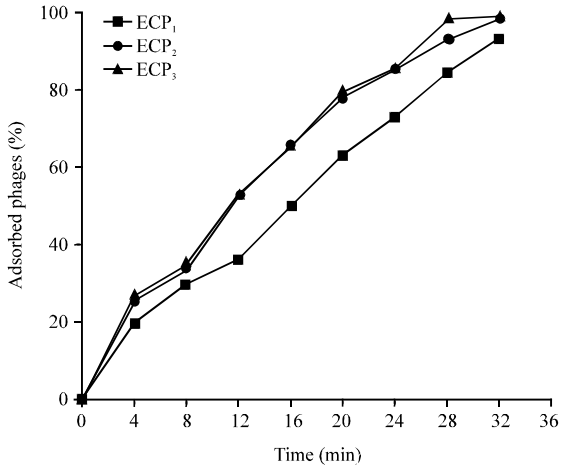

Fig. 5: Adsorption rate of the isolated coliphages

hydrophila (Table 1). These strains were used to study their susceptibility to either low or high titer of the three coliphages (ECP_{1-3}) using spot-test technique (drop method). Lytic areas were observed only when the coliphage ECP_1 spotted on the isolated bacteria *E. coli* W_1 and when coliphages ECP_2 and ECP_3 spotted on the isolated bacteria *E. coli* W_2. On the contrast, lytic areas were not observed with any other bacteria tested. Therefore, the ECP_1 was highly specific to infect the isolated bacteria *E. coli* W_1 and ECP_2 and ECP_3 were highly specific to infect the isolated bacteria *E. coli* W_2.

Adsorption experiment: The isolated coliphages ECP_1, ECP_2 and ECP_3 exhibited different adsorption rates (Table 2 and Fig. 5). Where coliphage ECP_3 had the highest value of the rate of adsorption constant (3.50×10^{-6} PFU/CFU/mL). On the other hand, coliphage ECP_2 had value of rate of adsorption constant (3.43×10^{-6} PFU/CFU/mL) and coliphage ECP_1 had value of

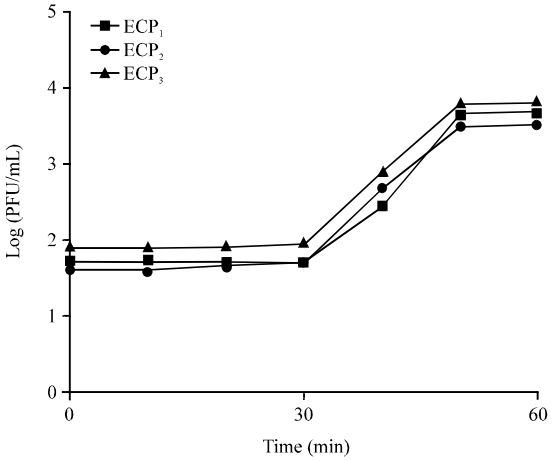

Fig. 6: One-step growth curve experiment of the isolated coliphages

Table 2: Growth characteristics of the isolated coliphages

Phage characters	ECP_1	ECP_2	ECP_3
Time for maximum adsorption (min)	32.00	32.00	32.0
Adsorption rate constant (PFU/CFU/mL)×10^{-6}	2.99	3.43	3.5
Maximum phage adsorption (%)	93.30	98.40	99.3
Latent period (min)	30.00	30.00	30.0
Rise period (min)	30.00	30.00	30.0
Burst size (PFU/Cell)	70.70	43.00	80.6

rate of adsorption constant (2.99×10^{-6} PFU/CFU/mL). All coliphages had the same time for maximum adsorption (32 min). About 80, 79 and 64% of all infective ECP_3, ECP_2 and ECP_1 particles were adsorbed within 20 min after contact, respectively. Moreover, coliphage ECP_3 had the highest value of maximum adsorption of about 99.3%. On the contrast, coliphage ECP_1 had the lowest one (93.3%).

One-step growth curve: The three coliphages of the current study ECP_1, ECP_2 and ECP_3 had almost identical latent period (30 min) during which no increase in phage titer was observed (Fig. 6). After latent period the titer of the coliphages increased until reached the maximum value during the rise period. Also, the three isolated phages had almost identical rise period (30 min).

The burst size indicated the mean number of coliphage progeny released per infected bacterial cell Stansfield *et al.* (1996). The burst sizes of ECP_1, ECP_2 and ECP_3 were 70.7, 43.0 and 80.6 PFU/cell, respectively.

Biocontrol of *E. coli* W_1, W_2 by their specific coliphages: As given in Fig. 7, mixing of cells of *E. coli* W_1 with their specific coliphage ECP_1 and mixing of cells of *E. coli* W_2 with their specific coliphages ECP_2, ECP_3 decreased viable cell population of host bacteria from almost 3.2×10^7, 2.1×10^7, 2.1×10^7 to 1×10^5, 1×10^5, 5×10^5 CFU mL^{-1}, respectively within 48 h but coliphages titers increased about 2.5 log cycles in the three cases.

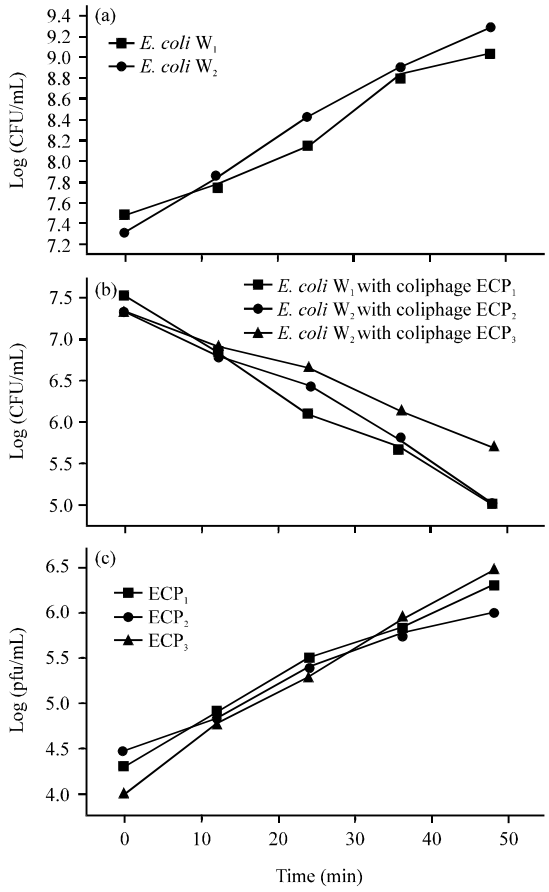

Fig. 7: a) Growth (CFU/mL) of the indicator *E. coli* W_1 and *E. coli* W_2 in nutrient broth without coliphages; b) and in nutrient broth with coliphages and c) titers (PFU/mL) of the three coliphages ECP_1, ECP_2 and ECP_3 appeared in BHI broth inoculated with their specific bacteria

DISCUSSION

Bacteriophages represent the largest of group all virus groups. They occur in archaea and bacteria and are found in enormous numbers in many diverse natural habitats. Phages are easy to isolate and inexpensive to purify (Ackermann and DuBow, 1987).

In this study, researchers isolated and characterized three coliphages from sewage water specific for *E. coli* W_1 and *E. coli* W_2. Electron microscopic examination showed that the three coliphages were classified as group A phages according to classification of Bradley (1967) and were belonging to the Myoviridae family according to classification of Mathews (1982). The coliphages ECP_1

and ECP_2 had isometric hexagonal head (50×40 and 90×70 nm, respectively) while coliphage ECP_3 had elongated hexagonal head (120×100 nm) and the three coliphages had contractile tails (40×10, 80×10 and 200×40 nm for ECP_1, ECP_2 and ECP_3, respectively). The ECP_3 coliphage had long tail with base plate and tail fibers. Therefore, these three coliphages resembled three phages S-BM1, S-PM1 and S-WHM1 of Wilson *et al.* (1993), two phages Aeh1 and KVP40 of Tetart *et al.* (2001), AR1 and LG1 phages of Goodridge *et al.* (2003) and ΦRSL1 phage of Yamada *et al.* (2010).

The infectivity of the isolated coliphages ECP_1, ECP_2 and ECP_3 decreased with the increase of the host age. The infectivity of ECP_1 and ECP_3 coliphages were more sensitive to the age of the host, its infectivity reached 25.2 and 23.6%, respectively after 96 h of host age. On the contrast, coliphage ECP_2 was the most resistant to host age where its infectivity reached 58.8% after 96 h of incubation.

The results of host experiment revealed that the tested coliphage ECP_1 exhibited specificity to *E. coli* W_1 while the tested coliphages ECP_2 and ECP_3 exhibited specificity to *E. coli* W_2. Therefore, these coliphages were similar to three phages of El-Helali (2001) who reported that phage Φ1C2 was specific for *Rhizobium leguminosurum* biovar *viceae* strain S while the two phages Φ2A and Φ4C specific for strain E. Moreover, these results were similar to phages of Singh *et al.* (1986) who found that the two phages PSP13 and PSP14 were specific to *R. solanacearum*. On the contrast, Eayre *et al.* (1995), Park *et al.* (1997), El-Sawi (1998) and El-Sayed *et al.* (2001) studied polyvalent phages which were active against more than one host.

Since, the tested coliphage ECP_1 exhibited specificity to *E. coli* W_1 while the tested coliphages ECP_2 and ECP_3 exhibited specificity to *E. coli* W_2. This study suggested use of these coliphages to identify *E. coli* strains. This suggestion was confirmed by Douglas (1975), Loessner and Busse (1990) and Sekaninova *et al.* (1998) who suggested the use of phages as tools for identifying bacteria (phage typing).

Isolated coliphages exhibited different adsorption rate constants where coliphage ECP_3 had the highest value of rate of adsorption constant (3.50×10^{-6} PFU/CFU/mL). On the other hand, coliphage ECP_2 had value of rate of adsorption constant of about 3.43×10^{-6} PFU/CFU/mL and coliphage ECP_1 had value of rate of adsorption constant of about 2.99×10^{-6} PFU/CFU/mL. All coliphages had the same time for maximum adsorption (32 min). About 80, 79 and 64% of all infective ECP_3, ECP_2 and ECP_1 particles were adsorbed within 20 min after contact, respectively. Moreover, coliphage ECP_3 had the highest value of maximum adsorption (99.3%). On the contrast, coliphage ECP_1 had the lowest one (93.3%). So, the tested coliphages exhibited high rate of adsorption since they reached maximum adsorption values after 32 min. Adsorption was therefore, one of the restricting factors. Anne *et al.* (1990) found that efficient adsorption (>90%) of VWB phage to *S. venezuelae* ETH 13640 and to *S. exfoliates* was probably due to particular phage receptor protein at the cell surface of these strains. They reported that about 70% of all infective VWB particles were adsorbed within 20 min after contact and the adsorption constant (K) was 0.6×10^{-8} mL min^{-1}. El-Tarabily *et al.* (1995) observed that the adsorption rate constants of the phages, ΦS1, ΦS2 and ΦS3 were 1.58×10^{-7}, 1.26×10^{-7} and 5.97×10^{-9} mL min^{-1}, respectively.

Isolated coliphages had the same latent period (30 min) and the same rise period (30 min) the length of these periods is not exceptional, they rather are suitable compared to those of many other phages (Lomovskaya *et al.*, 1980). El-Tarabily *et al.* (1995) reported that latent period values of ΦS1, ΦS2 and ΦS3 were 35, 40 and 40 min and the rise periods of the three phages were 40, 30 and 40 min, respectively. The phage, ΦA7 in *Streptomyces antibioticus* had a latent period of about 60 min and an exponential growth period of about 35 min (Diaz *et al.*, 1991). Anne *et al.* (1990) reported that the phage (VWB) infecting *S. venezuelae* has a latent period of 140 min followed by a rise period of 100 min. Rodriguez *et al.* (1986) found that the one-step growth curve of ΦC31 in 7 h germinated spores of *S. coelicolor* 01 has a latent period of about 45 min followed by a rise period of 20-30 min. The burst size values were different for the three coliphages where coliphage ECP_3 had the highest burst value (80.6 PFU $cell^{-1}$) and coliphage ECP_2 had the lowest corresponding value (43.0 PFU $cell^{-1}$). The burst sizes of coliphages of current study were more than that obtained by El-Tarabily *et al.* (1995) where he mentioned that the burst sizes of phages ΦS1, ΦS2 and ΦS3 were 15.5, 22.5 and 12.9 PFU $cell^{-1}$, respectively.

CONCLUSION

Inhibition of the *E. coli* W_1, *E. coli* W_2, *E. coli* W_2 by ECP_1, ECP_2 and ECP_3, respectively noticed in this study *in vitro* is a promising result to use coliphages as biocontrol and therapeutic agents. This result concur with previous results in this respect (Weinbauer, 2004; Enan *et al.*, 2012a, b; Abdallah *et al.*, 2013). Further, research will be necessary to study the inhibition of *E. coli* bacterium by its coliphages *in vivo* and in water.

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