

Antibiotics and Phage Sensitivity as Interventions for Controlling *Escherichia coli* Isolated from Clinical Specimens

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<http://dx.doi.org/10.22207/JPAM.11.4.13>

(Received: 03 October 2017; accepted: 09 November 2017)

Escherichia coli is considered one of the most frequent causative agents of common bacterial infections worldwide. In addition, effective treatment and prevention measures are still in demand due to the rise of antibiotic resistance and the emergence of new virulent strains. In this work, we evaluated antibiotics and bacteriophages as interventions for controlling pathogenic *E. coli*. A total of 15 *E. coli* isolates were included in this study. The automated identification system, namely Vitek 2, has been utilized for the identification. Antibiotics susceptibility profiles of all isolates were confirmed by disc diffusion method. All strains exhibited resistance at least to one antibiotic (ampicillin) while 13 strains were resistant to Ampicillin/Sulbactam, Cefazolin, and Ceftriaxone. Except for two strains, no resistance to Amikacin was observed. On the other hand, bacteriophages designated øEU-3 and øEU-4 were isolated by single plaque isolation and investigated as antimicrobial agents against pathogenic *E. coli*. Phages morphology, determined by transmission electron microscopy, revealed a structure comprised of a head diameter (71.42 nm) and a tail length (214.28 nm). These features placed the øEU-3 phage in the family *Siphoviridae* while øEU-4 belonged to family *Myoviridae* with a head diameter (66.6 nm) and a contractile tail length (108.3nm). Phages susceptibilities were determined by spot test to fifteen *E. coli* isolates. Coliphage øEU-3 and øEU-4 had narrow host range. This work described the efficacy of antibiotics and bacteriophages as intervention strategies to control pathogenic *E. coli* and paved the way for depth studies to broaden the antimicrobial spectrum of øEU-3 and øEU-4 phages.

Keywords: *Escherichia coli*, Antibiotic resistance, Coliphage, *Siphoviridae*, *Myoviridae*.

Escherichia coli is a gram-negative bacterium that is considered a part of the normal flora inhabiting the intestine and is thought to be harmless. However, pathogenicity mechanisms have been exhibited by many strains making them able to cause diseases in animals and humans. These diseases can be categorized into extra-intestinal (urinary tract infections (UTI),

meningitis, septicemia, and pneumonia) and intestinal (diarrhea)¹. Six pathotypes of *E. coli* causing intestinal diseases are known based on the pathogenicity profiles (clinical disease and virulence factors) and include enterohaemorrhagic *E. coli*, enteropathogenic *E. coli*, enteroinvasive *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, diffusely adherent *E. coli* and the recently emerged; adherent invasive *E. coli*²⁻⁴. The spread of prolonged-spectrum beta-lactamase producing *E. coli* capable of resisting several antibiotics and even the emergence of resistance to drugs of last

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resort such as colistin has become a major health threat worldwide and motivated the challenge for antimicrobial alternatives such as bacteriophages⁵⁻⁷.

Bacteriophages are bacteria-specific viruses⁸. Félix d'Hérelle, a co-discoverer of bacteriophages, was the first to propose "phage therapy"^{9,10} in the early twentieth century, however, the advent of antibiotics overtook the interest in phage therapy in that time. Nevertheless, phage therapy is now back in the headings¹¹ because of the emergence of new infectious antibiotic-resistance strains. *E. coli* phages, termed T-even, T-odd and Lambda phages, are a group of ds-DNA bacteriophages that have a head capsid and tail. Phages of *E. coli* were firstly isolated from a raw sewerage by T.L. Rakietyen in the late 1930s, and currently, they are the most studied among bacteriophages. They are commonly isolated from fecal samples, sewage, polluted rivers and hospital wastewater. Life cycles of *E. coli* phages could be lysogenic or lytic; however, for phage therapy, only lytic phages are used¹². Currently, phage therapy is being used in Russia, Georgia, Poland, and U.S.A

The current manuscript was designed to isolate and identify Gram-negative bacilli from Sohag University Hospital, Sohag Governorate, Upper Egypt, and to evaluate their antibiotics sensitivity patterns, also we tried to isolate host-specific bacteriophages from sewage water to evaluate the efficacy of phage therapy against the isolated *E. coli*.

MATERIAL AND METHODS

Samples collection and bacterial identification

The present study was done in Sohag University Hospital; a teaching hospital in Upper Egypt, with more than 1000 bed capacity, and Faculty of Science, Benha University, Qalubiyah governorate. Clinical specimens from the intensive care unit (ICU) were collected. Respiratory (R) secretions and tracheal aspirates (from intubated patients) were collected from patients with respiratory symptoms, urine (U) samples were collected aseptically in a sterile syringe from the demarcated site of catheterized patients, and swabs from surgical site infections (SSI) of patients were collected from deep incisions after skin antisepsis. The clinical specimens were collected under aseptic conditions and transferred immediately

for culture and sensitivity during the period from March 2016 to February 2017. All Samples were collected aseptically after 48 hours admission in ICU; transferred immediately to be inoculated on routine culture media and incubated aerobically at 37°C for 24 hours. Identification and antimicrobial sensitivity of the isolated gram-negative bacilli were done by Vitek 2 system.

Antibiotic susceptibility testing

Gram-negative identification and antimicrobial susceptibility testing cards were used to determine the susceptibility of the isolates to different antimicrobial agents. The isolates were either susceptible, intermediate susceptible or resistant to the antimicrobials. Susceptibility testing was confirmed using the disc diffusion (modified Kirby Bauer) method for the following antibiotics (Oxoid, UK); Ampicillin (AMP 10 µg), Ampicillin/Sulbactam (SAM 20 µg), Amikacin (AK 30 µg), Aztreonam (ATM 30 µg), Cefazolin (CZ 30 µg), Cefepime (FEP 30 µg), Ceftriaxone (CRO 30 µg), Ciprofloxacin (CIP 5 µg), Ertapenem (ETP 10 µg), Meropenem (MEM 10 µg), Gentamicin (CN 120 µg), Tobramycin (TM 10 µg), Moxifloxacin (MXF 5 µg), Nitrofurantoin (F 300 µg), Trimethoprim/Sulphamethoxazole (SXT 25 µg), Tigecycline (TGC 15 µg), and Imipenem (IPM 10 µg). The results were inferred according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013).

Demonstration of Sewage *E. coli* phages

Samples of sewage water were collected in sterile dark containers from the sewage water treatment plant at Benha, Qalubiyah Governorate, Egypt. The sample was first filtered through coarse filter paper to remove the debris, then the filtrate was serially diluted using 0.85% sterile saline, subsequently, the samples were filtered using 0.45 µm filters and stored at 4°C.

Isolation and propagation of *E. coli* phages

Fifty milliliters of filtered wastewater samples mixed with 50 ml of Luria Bertani (LB) broth inoculating with the log phase cells of *E. coli*. The culture media were incubated at 37°C for 24 h with speed of 120 rpm for bacteriophage enrichment. The media were centrifuged and filtered through a 0.45 µm membrane filter. The presence of lytic phage in the filtrate was examined by using the overlay method as described later.

One hundred µl of the filtrate was mixed

with 300 μ l of log phase culture of *E. coli* isolates and incubated at 37°C for 30 min. The mixture was added into a 3.5 ml of molten LB soft top agar (0.75% agar) which was already cooled down to 50°C, mixed gently and poured onto LB agar plate. The plates were left to stand at room temperature for 30 min to allow the top agar to solidify. The presence of lytic phages was distinguished according to the shape and size of plaques formed after incubation of the plates at 37°C for 24hr. Purification and propagation of *E. coli* phages were done by single plaque isolates according to (13).

Phage preparations

Stock lysates for the *E. coli* phages candidates were prepared by incubation of purified phage with the host in LB broth supplemented with CaCl₂ (2 mM) for 24-48 h at 37°C with shaking, the cultures were centrifuged (6000 rpm, 10 min) then filter sterilized and the resulting crude lysate was stored at 4°C.

Phage titering

Serial dilutions of the phages stock lysates were prepared and aliquots of these dilutions were incubated with the host at room temperature for 30 min. The incubated aliquots were added to molten LB soft agar and overlaid on an LB agar plate. Plates were incubated at 37°C for 24 h and the resulting plaques forming units (PFUs) were quantified to determine phage titer.

Spot test

The spot assay was used to assess the prevalence and the bactericide ability of the

sewage water *E. coli* phages against all the 15 isolated *E. coli* strains and was repeated three times for each bacterium. Five microliters of 45 μ m filter-sterilized purified phages were spotted onto the surface of the plates with the different *E. coli* strains. The plates were left to dry and were inspected for lysis zones after an overnight incubation at 37°C. A positive spot test appeared as complete obliteration of the entire drop area, whereas a negative spot test resulted in the bacterial lawn growing normally in the region of the spot. positive samples in the spot tests were confirmed by plaque assay¹⁴.

Transmission electron microscopy (TEM)

Ten microliters of the purified phage lysate with a high titer were spotted onto carbon-coated grids and stained with 1% uranyl acetate. Phages particles were observed under a JOEL-JM-100-C Transmission Electron Microscope (TEM) (Japan Electron Optics Laboratory Co., Ltd.) in the Regional Center of Mycology and Biotechnology at Al-Azhar University, Egypt.

RESULTS

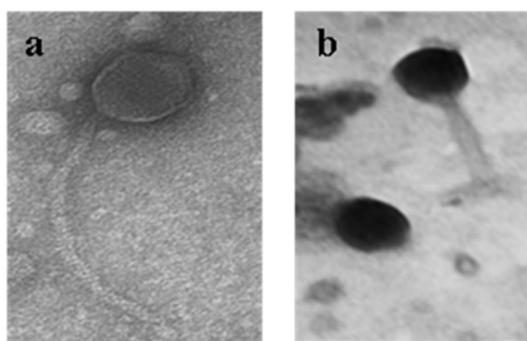
E. coli isolation and antibiotic susceptibility profiling

In this work, fifteen *E. coli* were isolated from clinical samples. They were designated as *E. coli* U-1 to *E. coli* U-4, *E. coli* R-1, *E. coli* SI-1

Table 1. Antibiotic resistance patterns of clinical *E. coli* isolates

Sample source	Isolate	øEU-3	øEU-4	Two phages cocktail
U	<i>E. coli</i> U-1	-	-	-
U	<i>E. coli</i> U-2	-	-	-
U	<i>E. coli</i> U-3	+	+	+
U	<i>E. coli</i> U-4	+	+	+
R	<i>E. coli</i> R-1	-	-	-
SSI	<i>E. coli</i> SI-1	-	-	-
SSI	<i>E. coli</i> SI-2	-	-	-
SSI	<i>E. coli</i> SI-3	-	-	-
SSI	<i>E. coli</i> SI-4	-	-	-
SSI	<i>E. coli</i> SI-5	-	-	-
SSI	<i>E. coli</i> SI-6	-	-	-
SSI	<i>E. coli</i> SI-7	-	-	-
SSI	<i>E. coli</i> SI-8	-	-	-
SSI	<i>E. coli</i> SI-9	-	-	-
SSI	<i>E. coli</i> SI-10	-	-	-

- denotes for resistant, +denotes for susceptible



100 nm
TEM Mag = 120000x

Fig. 1. Electron Micrographs of the isolate phages after negative staining of viral particles. (a) øEU-3 phage. (b) øEU-4 phage. The size bar corresponds to 100 nm

Table 2. In vitro susceptibility of different *E. coli* strains against sewage phages using spot assay

Sources of specimens	Isolate	AMP	SAM	CZ	CRO	FEP	ATM	ETP	IPM	MEM	AK	CN	TM	CIP	MXF	TGC	F	SXT
U	<i>E. coli</i> U-1	R*	R	R	R	R	R	S**	S	S	S	S	S	S	S	S	S	S
U	<i>E. coli</i> U-2	R	R	R	R	I†	I	S	S	S	S	R	R	R	R	S	S	R
U	<i>E. coli</i> U-3	R	NA	NA	I	NA	NA	NA	NA	NA	R	NA	NA	R	NA	NA	NA	NA
U	<i>E. coli</i> U-4	R	NA	NA	R	NA	NA	NA	NA	NA	R	NA	NA	R	NA	NA	NA	NA
R	<i>E. coli</i> R-1	R	R	R	R	R	R	S	S	S	S	S	S	R	R	S	I	R
SSI	<i>E. coli</i> SI-1	R	R	R	R	R	R	R	R	R	S	S	R	R	R	S	R	S
SSI	<i>E. coli</i> SI-2	R	R	R	R	R	NA††	NA	NA	S	S	R	R	R	NA	NA	S	R
SSI	<i>E. coli</i> SI-3	R	R	R	R	S	NA	NA	NA	S	S	R	R	R	NA	NA	R	R
SSI	<i>E. coli</i> SI-4	R	R	R	R	R	R	S	S	S	S	S	R	R	R	S	S	S
SSI	<i>E. coli</i> SI-5	R	R	R	R	R	NA	NA	NA	S	S	S	S	S	NA	NA	S	R
SSI	<i>E. coli</i> SI-6	R	R	R	R	R	NA	NA	NA	S	S	R	R	R	NA	NA	NA	R
SSI	<i>E. coli</i> SI-7	R	R	R	R	S	I	S	S	S	S	S	S	S	S	S	S	R
SSI	<i>E. coli</i> SI-8	R	R	R	R	R	NA	NA	NA	R	S	S	R	R	NA	NA	S	S
SSI	<i>E. coli</i> SI-9	R	R	R	R	R	NA	NA	NA	S	S	R	R	R	NA	NA	NA	R
SSI	<i>E. coli</i> SI-10	R	R	R	R	R	NA	NA	NA	S	S	R	R	R	NA	NA	S	R

*denotes for Resistant, **denotes for Susceptible
 † Intermediate †† Not tested

Table 3. Dimensions and titration of the isolated phages

Phage (PFU/ml)	Prospected Family	Head diameter (nm)	Tail length (nm)	Phage titer
øEU-3 phage	<i>Siphoviridae</i>	71.42	214.28	2 x 10 ⁷
øEU-4 phage	<i>Myoviridae</i>	66.6	108.3	2 x 10 ⁵

to *E. coli* SI-10 as they were isolated from the urine samples, respiratory infections and surgical site infections, respectively. All strains were resistant to at least one antibiotic (ampicillin). Thirteen strains were also resistant to Ampicillin/Sulbactam, Cefazolin, and Ceftriaxone. Except for two strains, no resistance to Amikacin was observed. In addition, all strains were sensitive to Meropenem except for two strains. Nine strains were resistant to Aztreonam and six strains resist to gentamycin. One strain was resistant to Ertapenem and Imipenem while nine and twelve strains resisted to tobramycin and Ciprofloxacin, respectively. Furthermore, nine strains were resistant to Trimethoprim/Sulphamethoxazole and out of six strains investigated for Tigecycline, no resistance was established. The antibiotic resistance patterns of all strains were summarized in Table 1.

Bacteriophage susceptibility

To investigate the prevalence of bacteriophages in municipal sewage water against the previously isolated bacteria, a spot technique was performed, and the results were summarized in Table 2. Out of the fifteen isolated *E. coli* strains, only two were shown to be sensitive to sewage bacteriophages, *E. coli* U-3 and *E. coli* U-4, as judged by spot test and later by plaque assay. The spot technique served as both an indicative experiment of bacteriophages presence and gave an idea of the host range of the resulted phages (data not shown), single isolated phages, as well as mixed cocktails of the two phages, were used in the preceding experiments. The accumulated data revealed a narrow-host-range of the isolated phages against the fifteen isolates. Single plaques were picked from the plates with visible clear plaques, from *E. coli* U-3 and *E. coli* U-4 lawns, the plaques were then propagated in liquid cultures. The previous phages, named øEU-3 and øEU-4 phages, were selected for imaging with transmission electron microscope.

Morphology and diameters of øEU-3 and øEU-4 phages are shown in Fig.1 and Table3. Electron micrographs of øEU-3 phage showed an icosahedral head with a diameter of ~71.42 nm and a 214.28 nm long non-contractile tail (a character of *Siphoviridae* phages). Micrographs of øEU-4 phage showed that it has an icosahedral head with a diameter of ~66.6 nm and a 108.3 nm long contractile tail (a character of *Myoviridae* phages).

DISCUSSION

Over the last decades, the prevalence of multidrug-resistant bacteria, particularly *E. coli* has gained a great interest due to their increasing rates on a daily basis¹⁵. The occurrence of extended-spectrum Beta-lactamase producing *E. coli* could be nearly 63% in many countries while the highest rates belong to *E. coli* from ICU patients^{16, 17}. Our work indicated highly drug-resistant *E. coli* could be present in the ICU. Many *E. coli* isolates showed resistance to at least five antibiotics. In previous studies, high resistance to antibiotics has been described for *E. coli* isolated from UTI samples¹⁸. Moreover, high frequency of *E. coli* resistance to multiple antibiotics such as penicillin, co-trimoxazole, and nitrofurantoin has been documented¹⁹. In another study, 170 (43%) of *E. coli* isolates were multidrug resistant and showed high resistance to Ampicillin (82.53%), Amoxicillin-clavulanic acid (71.90%), Ceftriaxone (66.58%), Ciprofloxacin (65.82%)²⁰. It was indicated that of total 328 strains of *E. coli*, 199 strains exhibited higher resistance rate to ampicillin, tetracycline, trimethoprim/sulfamethoxazole and cefazolin, and 34.6% of these *E. coli* strains exhibited resistance to at least four antibiotics. Three strains were resistant to seven antibiotics in the same study. The treatment of multidrug-resistant pathogens continues to be a challenging problem. Sustainable and effective alternative approaches have become a necessity. One of the most popular approaches is employing bacteriophages used as anti-infective agents to circumvent antibiotic resistance.

Phage therapy has various merits over antibiotics: (1) bacteriolysis mechanisms by phages differ from antibiotics mechanisms (2) so, it is very effective against antibiotic-resistant bacteria; (2) bacteriophages are very specific so, they will be harmless to other beneficial bacteria in human guts; (3) Phages undergo mutations so, they can respond quickly to phage-resistant mutants; (4) ease of isolation; (5) no side effects are known so far; (6) high therapeutic index, smaller effective dose, and a single shot is required; (7) production of phages are very quick and cheap as compared to development of a new antibiotic^{22, 23}. The major obstacles against phage therapy are legislation which might take years to be approved. Currently, Russia, Poland, Georgia, China and U.S.A are

using bacteriophages to treat systemic and enteric diseases that do not respond to conventional antibiotics. Bacteriophages are ubiquitous in different watersheds throughout the world and proposed to be most copious biological objects in the biosphere^{24,25}. Bacteriophages existed since the bacterial cells originated, wherever the bacteria are found, bacteriophages were found associated. In current study, we succeeded to isolate lytic bacteriophages, from sewage water, against two of the preceding *E. coli* isolates. Likewise our study, many researchers have isolated phages against *E. coli* from sewage²⁶. The morphology of isolated *E. coli* phages, designated bacteriophages øEU-3 and øEU-4, showed that they belong to the family *Siphoviridae* and *Myoviridae*, respectively. Similar study reported isolation of *E. coli* siphophages from stool of pediatric patients with a complaint of diarrhea²⁷ and others indicated in healthy subjects, as lambda-like *Siphoviridae* phages were mainly isolated from stool samples of healthy persons^{28,29} while stools of diarrhea patients gave predominantly T4-like *Myoviridae*. But when using different indicator cells, different phages were also isolated from the same stool samples²⁷. Our results showed that the two isolated phages (øEU-3 and øEU-4) were investigated for their potential use as antimicrobial agents against the isolated multidrug resistant clinical *E. coli*, whereas, *E. coli* U-3, *E. coli* U-4 isolates were susceptible to phages cocktail infection, but the rest of studied isolates revealed different patterns of resistance to antibiotics as well as phage cocktail which displayed a narrow host range.

CONCLUSION

This study provided evidence that *E. coli* isolated from clinical specimens although being sensitive to certain antibiotics, they exhibited resistance to a wide range of other drugs. In addition, the use of lytic bacteriophage cocktail as antimicrobial agent against the isolated *E. coli* strains revealed the efficacy of the two isolated phages to control two out of fifteen strains used in this study and paved the way for future studies to focus on expanding their antimicrobial spectra by combining them with other antimicrobials and/or newly isolated phages.

ACKNOWLEDGEMENT

Funding for this study was partially supported by a grant from the Scientific Research Fund (SRF) from Benha University (A.G.A), other funds were from the Egyptian Ministry of Higher Education and Scientific Research.

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