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Enzybiotics Activity of Phage Endolysin for Treatment of Multi-Drugs Resistant *Escherichia coli* UTI: *in vitro* and *in vivo* study

Haider N. Saqi[1*], Ahmmad S. Abdul Ameer[2]

Kirkuk Health Directorate-Iraq[1]
College of Medicine- Al-Naharin University-Iraq[2]

Corresponding author: 1*

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ABSTRACT

Resistant infection with multidrug-resistant *Escherichia coli* (*E. coli*) is one of the most common causative agents of bacterial infections and emergence as a multidrug resistant bacterium is a major public health threat worldwide and representing a real problem for health care providers. The use of Bacteriophage lytic enzymes (lysins) are highly evolved molecules that have been specifically developed by phages to quickly and efficiently allow their progeny to be released from the host bacterium while destructing that bacterium. This study aimed for isolation of endolysin from *E. coli* bacteriophages, and administering them systemic *in vivo* lab animal and measure the therapeutic efficacy, as well as evaluation of their biosafety for treatment of multi-drugs resistant *Escherichia coli* in urinary tract infection. This study was performed from September 2019 to February 2020, up to 30 bacteriological samples of uropathogenic *Escherichia coli* were collected, and examined with their anti-biogram, then bacteriophage cocktails were prepared for three resistant strains of them. Endolysins were extracted from their corresponding bacteriophages, they were characterized; the enzymatic and antibacterial activities of extracted endolysins were tested by *in vitro* assays; in addition, *in vivo* experiment was carried out on rabbit animals regarding the therapeutic effect of these extracted endolysins on UTI-causing *E. coli*. This study showed that the extracted endolysin from highly lytic coliphages were successful in reducing bacterial count in urine of treated rabbit animals which received specific endolysin via venous catheter. Endolysin can be extracted directly from their bacteriophages and being purified and used. The injection of rabbits with UTI with the proper dose of the extracted endolysin was effective in eradicating UTI in all of the treated animals.

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1. Introduction

Antimicrobial resistance (AMR) including antibiotic resistant is a serious public health problem, which

became an issue of global concern. It had been predicted that its attribute mortality will hit 10 million by 2050 if measures are not taken to tackle it. ABR represents one of the biggest challenges to global public health, particularly, with the development of known, and unknown resistance mechanisms [1].

The emergence of drug-resistant pathogens that have acquired new resistance mechanisms, led to threatening our ability to treat common infections. Also, the possibility of spread of multi- and pan-resistant bacteria "superbugs" can cause infections that are not treatable with existing antimicrobial medicines [2].

Antimicrobials are probably one of the most successful forms of chemotherapy in the history of medicine and played a major role in the fight against infectious diseases [3]. Bacterial resistant to antibiotics can limit effective treatment, rendering bacterial infections difficult to treat, including Urinary tract infections (UTI) [4]. Bacteriophage depolymerase plays an important role in the degradation of biofilm Extracellular Polymeric Substances (EPS) substrate, promoting phage penetration into the biofilm and leading to bacterial cell lysis [5].

Indeed, this enzyme is expressed on the surface of phage capsids or produced by host cells during phage replication and it helps phages to adsorb, attack, and decompose bacterial host [6]. Furthermore, phages at the end of the lytic cycle produce endolysins. Endolysins are phage enzymes that cleave peptidoglycans, i.e., the main component of the bacterial cell wall, and they are antibacterial agents owing to their special mode of action and highly particular activities against bacteria [7].

Interest in bacteriophages has increased since the late 20th century as a response to the emergence of multidrug-resistant bacteria [8]. The lytic replication of bacteriophages requires the insertion of phage's genetic material into the bacterial host, resulting in virion multiplication and, for most bacteriophages, the expression of endolysins, proteins that cause cell lysis throughout the disruption of peptidoglycan [13], 14]. Cell lysis is caused by phage endolysins and other accessory proteins such as holins and pinholins, that act in the cytoplasmic membrane, and spanins, that form junctions between the inner and outer membranes of Gram-negative bacteria [9], [10].

Phage endolysins are subdivided into (I) glucosaminidases; (II) lysozymes or muramidases; (III) lytic transglycosylases; (IV) endopeptidases; and (V) amidases; depending on the peptidoglycan disruption mechanism [11]. The cleavage of a conserved structure such as the peptidoglycan points to endolysins as promising antimicrobials against multidrug-resistant Gram-negative bacteria [12]. Bacteriophage endolysins are active from the inside of the cell. Bacteriophage-encoded accessory proteins help cytoplasmatic membrane export to reach the peptidoglycan [13], [14]. Despite some advantages over the use of bacteriophages, such as broad-spectrum specificity and no report of bacterial resistance, the exogenous application of endolysins against Gram-negative bacteria is difficult due to the low permeability of the outer membrane and some endolysins require additional strategies to permeate the outer membrane [15], [16].

2. MATERIALS AND METHODS

2.1 Bacterial sampling

Samples of bacteria were collected in Kirkuk General Hospital. Bacterial sampling was carried out during the period from September 2019 to Febraury 2020. A total of (30) different *Eherichia coli,* isolates, belonging to hospitalized patients and outpatients suffering from severe urinary tract and recurrent urinary tract infections were obtained from the central laboratory of the Kirkuk hospital. The specimens were cultured in screw universal tubes containing nutrient broth covered by parafilm or by using sterile swabs;

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both were put in ice bags and were transported, at the same day, to general health laboratory of Kirkuk to subculture bacteria on nutrient and MacConkey agar or to be stored in refrigerator at 4^0C for 24h.

After storing the bacterial isolates, at sterile conditions, for 24h at 4^0C in a refrigerator, specimens were inoculated on blood agar and on MacConkey by streaking method, and the plates were incubated at 37^0C for 18-24h in an incubator. *Eherichia coli* formed on MacConkey'smedium-pink due to lactose fermentation, circular, moist, smooth, with entire margin, non-mucoid colonies. Solitary colonies of *Eherichia coli* were isolated from a growing stock by ABC streaking on MacConkey agar plates in order to isolate only discrete colonies. Then, single colony was sub-cultured on MacConkey agar and incubated for another 24h at 37ºC. All of bacterial isolates were examined for gram stain ability and conventional biochemical tests including, Oxidase test, Catalase test, ferment most of the sugars (glucose, lactose, mannitol, maltose) with acid and gas. Typical strains do not ferment sucrose, Indole and methyl red (MR) reaction are positive but Voges-Proskauer(VP) and citrate utilisationtests are negative (IMVic++--) ,Urease–ve, Gelatin not liquified, H2S not formed [17], [18].

Identification results were confirmed by API 20E system. The media were prepared according to the manufacturing instructions and were sterilized by autoclaving at 121^0C for 15min. The media were cooled to 45^0C and were then poured into sterilized petri dishes. The specimens were inoculated on plates. Then, plates were incubated in an incubator at 37^0C for 18-24h. Next day, bacterial growth was examined by API biochemical test for confirmation. Finally, confirmed *Eherichia coli* isolates were sub-cultured in nutrient broth containing glycerol (30% v/v), and were stored at -20^0C.

2.2 Sampling and processing of Bacteriophages

Sewage samples for bacteriophage isolation were obtained from different regions in Kirkuk and Baghdad cities including sewage, farm soil, waste water, feces of sheep and chicken litter in (Al-Jihad Quarter, Al-Imamein Al-kadhimein Medical City Hospital) from November 2019 to May 2020. The crude samples were obtained (50ml) in clean test tubes then were enfolded by parafilm, held in ice bag and were transported to the laboratory to be processed at the same day.

Primary phages are those phages that were isolated from environmental specimens when were mixed with target bacteria (*E coli*). The procedure of isolating and propagating primary phages was done according to the methodology conducted in a recent patent [19] and as follows:

Bacterial stocks were prepared by growing bacteria overnight on nutrient broth. About 100 µl of 10 bacterial isolates were mixed together in a sterile 50 ml test tube. Then, 2–3 ml of crude samples, which were derived from sewage, cattle feces, chicken litter, mastitis discharge swabs that might contain E coli specific phages were added to the mixture. Then, 2–3 ml, equal volume, of nutrient broth and 2 ml of Lambda buffer were added to the mixture as well. Then, the mixture was incubated overnight at 37°C. Next day, 5 ml of the crude mixture were dispensed into a sterile 15 ml test tube, centrifuged at 1,000 g for 3 min at room temperature. One ml of supernatant was transferred to 1.5 ml Eppendorf tube. Then, 1:10 v/v chloroform was added to the supernatant with gentle shaking for 7–10 min at room temperature to lyse the remaining bacteria. Centrifugation of the Eppendorf tubes at 1,000 g for 3 min; the supernatant was transferred into new Eppendorf tube and equal volume of lambda buffer was added. Thus, the primary phage suspension, if any, was produced.

Virulent phages were screened by phage spotting test on a nutrient-agar. Phage spotting can be used to provide a first approximation of the ability of a phage to lyse certain bacterial isolates. The formation of

clear zones suggested the presence of lytic phages. At first, the target bacteria were refreshed in nutrient broth at 37°C for 24 h. After overnight incubation, 200 µl of the bacterial broth were poured on to the nutrient agar plate to make bacterial lawn. After 2–3 min, the lawn should have been dried. Using a mechanical pipette, 10 µl of primary phage suspension were dropped on to the surface of the bacterial lawn and were allowed to dry before incubating at 37°C for 24 h. On the next day, a lytic and specific phage can be discovered for the target bacteria if zone of lysis was developed at the spot where the primary phage suspension was applied.

Then, specific lytic phages to MDR- *E coli* were picked up by sterile loop and put into 1 ml of Lambda buffer in 1.5 ml sterile Eppendorf tubes with gentle shaking for 5 min. About 1:10 v/v chloroform was added to the lysate with gentle shaking for 5–7 min at room temperature. Host cell debris was pelleted by centrifugation at 1,000g for 3 min, and the supernatant containing phages was transferred to 1.5 ml sterile Eppendorf tubes and stored at 4°C. The supernatant was called transient phage stock suspension.

2.3 Extraction of Endolysin

Up to 100 ml of broth containing bacteria (*E coli*) was incubated for 18 h at 37°C. Next day, the bacteria were put in 1 L of broth for 3 h (1 × 1012). A total of 300 ml of the bacteriophage at titer 1 × 1013 pfu/ml was added for 20 min (1:10 MOI) after dividing the total volume in 50 ml tubes and then were put directly in ice. They were centrifuged at 10,000 rpm for 15 min and the sediment was taken.

The sediment was put in 6 ml of 0.05 M phosphate buffer + deoxyribonuclease (5 mg). Then it was incubated for 60 min at 37°C. Ethylenediaminetetraacetic acid (EDTA) (0.005 M) was added and centrifuged at 10,000 rpm for 1 h and the supernatant was taken. Disodium tetrathionate (0.3 M) was added and mixed for 1 h at 4°C. Ammonium sulfate was added to 85% saturation and incubated for 18 h at 4°C. Next day, this was centrifuged at 10,000 rpm for 1 h. Then the mixture was resuspended in 5 ml of 0.05 M phos¬phate buffer (pH 6.1). Dialysis against 200 ml of the phos¬phate buffer saline (PBS) with (2×) conc. And pH = 6.1 at 4°C overnight was done. Then it was added to column chro¬matography Sephadex G100 in 0.1 M phosphate buffer (NaCl = 8 g/l, KCl = 0.2 g/l, Na2HPO4 = 1.4 g/l, KH2PO4 = 0.2 g/l) pH 6.1, in 18 × 0.5 cm column. They were collected in 0.5 ml Eppendorf tubes at 10 min intervals. From each Eppendorf tube, 10 µl was dropped by using automatic pipette to bacterial lawns of the specific bacteria to see which Eppendorf tube contain the endolysin, if any.

2.4 In vivo therapeutic experiment of the Extracted Phage Endolysin on clearing UTI in rabbit animals

Albino rabbits males of mean body weight 1.2 ± 0.1 Kg per rabbit and of age 4-6 weeks were used. The therapeutic effect of extracted endolysin was evaluated by using *E.coli* UTI-induced three rabbits versus three rabbits without endolysin treatment as control group. Rabbit animals were subjected to get artificial UTI and bacteria were introduced to base line of 5 ml of 7.8 x10^9 CFU/ml for bacterial infection using Urinary tract catheter. After 24 hr, samples of urine were collected from each rabbit for bacterial isolation and to confirm diagnosis of the inoculated MDR-*E coli* in urine of the rabbit animal by using MacConkey culture media. Later each one of infected rabbit received (0.5ml of 77ug/ml) specific phage endolysin via venous catheter. Rabbits were then monitored for their health and physical activities using specific health score and the bacterial count of E. coli in the urine collected by urinary catheter from treated animals and data was recorded periodically after (8 hr, 16 hrs, 24 hrs, 32 hrs, 40 hrs, 48 hr, 56 hr, 64hr, and 72hr).

3. FINDINGS AND DISCUSSION

The Endolysin was successfully extracted from all the three *E coli* bacteriophages by using Sephadex G100 column chromatography. The estimated endolysin concentration, after extraction and purification, was 77

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ug/ ml.

The findings of the current study revealed a remarkable reduction in the bacterial count (CFU/ml) in urine of endolysin-treated UTI-induced rabbits over 3 days (8 hr, 16 hr, 24 hr, 32 hr ,40 hr,48 hr,56 hr,64 hr and 72 hr) after injection of endolysin through venous catheter as shown in table 1. The three control rabbit animals showed 1, 1.5, and 1.2 log reduction only after 3 days of UTI induction. Hence, the use of specific endolysin for the inoculated *E. coli* causing UTI in rabbit animals resulted in time-dependent significant log reductions in the urinary bacterial count of E. coli as shown in table 2.

Table (1): Bacterial count (CFU/ml) of isolated bacteria from the rabbit's urine after endolysin injection.

Groups bacteria	Base line bacteria	Number Colony (CFU/ml)								
		Day 1			Day 2			Day 3		
		8 hr	16 hr	24hr	32hr	40 hr	48hr	56hr	64 hr	72 hr
Esherichia coli 1	**9.5×10^5**	3000	260	250	120	60	30	4	0	0
Esherichia coli 2	**8.9×10^4**	3500	310	290	93	40	20	3	0	0
Esherichia coli 3	**6.3×10^4**	2400	215	185	80	54	25	2	0	0

Table (2): The log reduction Bacterial count(CFU/ml) of isolated bacteria from the rabbit's urine after endolysin injection.

Groups Lysine	Log Reduction Bacterial Count(CFU/ml)								
	Day 1			Day 2			Day 3		
	8 hr	16 hr	24 hr	32 hr	40 hr	48hr	56hr	64 hr	72 hr
E_1 lysin	6.50	7.56	7.57	7.89	8.19	8.50	9.37	9.97	9.97
E_2 lysin	5.40	1.05	0.02	049	0.36	0.30	0.82	0.47	/
E_3 lysin	5.41	6.46	6.53	6.89	7.06	7.40	8.49	8.79	8.79

E1 lysine: Esherichia coli, E2 lysin: Esherichia coli, E3 lysin: Esherichia coli

Multi-Drug Resistant (MDR) uropathogenic bacteria have increased in number in recent years and the development of new treatment options for the corresponding infections has become a major challenge in the field of medicine. In this respect, recent studies have proposed bacteriophage (phage) therapy as a potential alternative against MDR Urinary Tract Infections (UTI) because the resistance mechanism of phages differs from that of antibiotics and few side effects have been reported for them. Escherichia coli are the most common uropathogenic bacteria against which phage therapy has been used phages, in addition to lysing bacterial pathogens, can prevent the formation of biofilms. Besides, by inducing or producing polysaccharide depolymerase. The present study conducted to reveal the of endolydin enzymes isolated from phages against MDR-*E. coli* [20].

The target specificity of endolysins renders it a promising antibiotic substitute, because endolysin is not toxic to the eukaryotic cell and does not disrupt environmental microorganisms and normal floral bacteria. Moreover, chemical antibiotics are of limited abundance and the rate of MDR development is higher than the rate of discovering new antibiotics. On contrary, endolysins are of unlimited source with much easier and quicker process to find new ones.

The findings of the current study showed a remarkable reduction in the bacterial count(CFU/ml) with high rates of log reduction of all of the isolated bacteria: *Eherichia coli* from rabbit's urine within 8, 16, 24, 32,40,48,56,64 and 72 hr after endolysin injection through venous catheter. The results of estimated endolysine concentration after extraction and purification from prepared bacterial broth of bacteria; *Esherichia coli* were endolysine concentration 77 ug/ ml.

This provided evidence for the feasibility to use endolysins as capable antimicrobials to treat UTI; moreover, this showed that endolysins pharmacokinetics are suitable for treating UTI and G-ve endolysins are well excreted in urine of rabbit animals. Hence, both orally-fed lytic phages and their parenterally-injected endolysins are shown to be effective in reaching urine and killing target G-ve bacteria. This allows more freedom for UTI management of MDR, XDR, or PDR uropathogenic bacteria causing UTI.

Different clinical studies demonstrated the safety of bacteriophages and bacteriophage lytic enzymes which is consistent with the extensive evidence on their safety in animal models and human and being able to target bacteria in both local and systemic infections.

Various studies suggest phage therapy has the potential to be used as either an alternative or a supplement to antibiotic treatments [21]. Bacteriophage depolymerase plays an important role in the degradation of biofilm Extracellular Polymeric Substances (EPS) substrate, promoting phage penetration into the biofilm and leading to bacterial cell lysis. Indeed, this enzyme I expressed on the surface of phage capsids or produced by host cells during phage replication and it helps phages to adsorb, attack, and decompose bacterial host [22].

Phage endolysins are enzymatic proteins responsible for cell wall degradation, bacteriophages use them to hydrolyze the peptidoglycan of the infected bacteria. Endolysins perform activities of endopeptidase, amidase, glycosidase or lytic transglycosylase to kill bacterial cell by murein destruction. Acting at the end of the phage replication cycle, endolysins promote the release of progeny virions [23].

Considering the *InVivo* therapeutic efficacy of endolysine three rabbit received bacteria; *Esherichia coli* and later each one of infected rabbit, received specific phage endolysine injection by venous catheter then rabbits were monitored for their health and physical activities and health score was recorded, in addition to the urine samples were collected by urinary catheter for bacterial isolation and the results referred to the (CFU/ml) bacteria count reduced gradually at the examined times due to endolysin activity.

It seems to be the reason why endolysins targeting gram-negative bacteria are small globular proteins composed of only one domain, called enzymatically active domain (EAD) [24]. Endolysins (lysins) are directly responsible for killing bacterial cells. They are produced at the end of the lytic replication cycle of dsDNA bacteriophages and accumulated within the host cell cytoplasm until they are activated. Due to their ability to cleave the peptidoglycan bonds, they disrupt the bacterial cell wall from the inside, thus enable releasing the virus progeny [25]. Lysins' highly conservative Nterminal domain has a catalytic activity and is responsible for enzymatic function, while the C-terminal domain, capable of specifically recognizing ligands within the bacterial wall, shows a great deal of variety [26].

4. Conclusion

Native endolysin extracted from highly lytic phages that produced halo-like appearance around inhibition zone was able to reduce bacterial viability (about 6.5 log) after 8 hours of treatment. Endolysin can be extracted directly from their bacteriophages and purified and used. The injection of rabbits with bacteremia

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with the proper dose of the extracted endolysin of the corresponding bacteriophages was effective in all of them.

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