

Characterization of Bacteriophages Specific for Multi-Drug Resistant *E. Coli* Isolated from Diabetic foot Patients

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ABSTRACT

Foot ulcers are the main cause of hospitalization and mortality of diabetic patients over the world. Approximately 25 % of diabetic patients will suffer from wound infections during their lifetime. Resistance of bacterial pathogens to available antibiotics has become a great load to the human health care, resulting in a significant progression of morbidity and mortality. Therefore, the use of novel solutions with antimicrobial properties such as bacteriophages attracted the attention of many investigators. In the current study, twenty seven multidrug resistant (MDR) *E. coli* isolates were isolated from hospitalized diabetic foot patients and bacterial identification was done based on biochemical and molecular characteristics using 16srRNA. Four bacteriophages with lytic activity against MDR *E. coli* were obtained. Two different bacteriophages were characterized physically through the evaluating of their lytic activities at a wide range of temperatures (40–90° C) and pH values (2–12) and biologically by evaluating their host ranges and the rate of adsorption. Transmission electron microscope (TEM) study was revealed that both phages belong to Myoviridae family. The present study opens a new window for the application of bacteriophages as promising antimicrobial agents against MDR bacteria.

Keywords

Diabetic foot, Multi drug resistant bacteria, *E. coli*, Bacteriophage

Introduction

Diabetes mellitus (DM) is commonly referred to diabetes which is considered a chronic disease and metabolic disorder that characterized by high glucose concentrations. DM has been categorized into two main types according to the International Diabetes Federation. Type 1 diabetes mellitus (T1DM) that known as insulin-dependent diabetes mellitus which is reported in about 10–15% of all diabetes mellitus and considered as an auto-immune disorder that mostly starts in infected persons before age 40 and characterized by the self - destruction of insulin-producing beta pancreatic cells by the body's own immune system. Type 2 diabetes mellitus (T2DM) formerly known as non-insulin dependent diabetes mellitus and also referred to as late-onset diabetes and accounts for the bulk of diabetes worldwide about 90% of all diabetic cases. The distinctive feature of this type is the relative insulin deficiency and resistance which lead to build-up plasma glucose [1]. At high risk, diabetic patients suffer from developing foot ulcers and subsequent microbial infection [2].

Escherichiacoli is reported as the main cause for 70% of patients with DM. There are a correlation between amyloid producing *E. coli* in the intestine, followed by their reduction by induction of prophage, and the initiation of autoimmunity and progression of diabetes especially Type 1 Diabetes mellitus [3]. Furthermore, high level of blood sugar has been associated with the rapid multiplication of *E. coli* followed by establishment of severe infection [3, 4]. *E. coli* is Gram negative rod-shaped of the family *Enterobacteriaceae*, facultative anaerobes and non-spore forming that commonly associated with digestive microflora and naturally excreted in faeces. *E. coli* can cause approximately 80% of cases of meningitis and 40% of cases of septicemia that lead to septic shock, fever, hypothermia or hypotension. In severe cases it may be complicated by uremia, hepatic failure, coma and finally death [5, 6]. Different strains of *E. coli* as Enteroinvasive, Enterotoxigenic and Shiga toxin-producing *E. coli* (*E. coli* O157:H7) are associated with distinctive diarrheal illnesses [7].

The appearance of multidrug-resistant (MDR) bacterial strains, particularly human pathogens, is one of the major problems of current medicine [8]. Phage therapy (PT) is reported to be one of the most important

alternative strategies to reduce or eliminate bacterial infections particularly the resistant ones to the action of antibiotics. In the last few years, significant works have been achieved concerning the application of bacteriophages as a promising therapy in the context of the rise of antimicrobial resistance (AMR) and can be considered as a potential replacement for antibiotics [9, 10].

The potential therapeutic option of bacteriophages is based on several advantages over conventional antimicrobial drugs regarding medical application. Bacteriophages are antibacterial agent that can regulate bacterial populations by the induction of bacterial lysis; they are highly numerous, usually readily isolated and active against Gram-positive as well as Gram-negative bacteria, including MDR pathogens. They have specificity against one single species or even strain of bacteria. These advantages of phage reduces the harmful effect on the natural microflora of the human body and avoided the side-effects of antibiotics [11]. Bacteriophage therapy is also suitable for use in humans, since bacteriophage does not infect cells of eukaryotic [12]. Another advantage is that phages replicate at the site of infection, ensuring their safety and absence of side effects, even after their wide distribution upon systemic administration. Unlike antibiotics, phages still infective under unfavorable environmental conditions [13].

The aim of the current study was to isolate bacteriophages specific for MDR *E. coli* isolated from diabetic foot infected ulcers and investigating of their physical and biological properties. Physical properties of bacteriophages include pH and thermal stabilities, while biological properties include host range, longevity and adsorption rate of isolated bacteriophages. The morphological characteristics were also studied using TEM.

Methodology

1. Isolation and Identification of *E. coli* isolates

A total of 85 selected diabetic foot samples were obtained from patients with foot ulcers hospitalized in the Sednawy Health Insurance hospital, Al Demerdash Hospital, Nasr City Health Insurance hospital, and different private diabetic foot clinics in Cairo (Egypt). The specimens were cultured onto blood agar and MacConkey's agar media (Oxoid), using the plate streaking technique [14]. The plates were incubated at 35–37 °C for 24–48 h. The isolated bacterial colonies were chosen and picked up according to culture characteristics then purified by successive sub-culturing on the same media as appropriate and stored at 4 °C till used. The bacterial isolates were identified according to their morphological characteristics, biochemically using Vitek2.

2. Antimicrobial susceptibility testing

E. coli isolates were tested for susceptibility to Ampicillin (10µg), Aztreonam (30µg), Carbenicillin (100µg), Cefaperazone Sulbactam (75+25µg), Cefazolin (30 µg), Cefuroxime (30 µg), Cefotaxime (30 µg), Cefepime (30µg), Ceftriaxone (30µg), Ceftazidime (30µg), Chloramphenicol (30 µg), Colistin (10 µg), Polymixin B (50 µg /300 IU), Ofloxacin (5 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Tobramycin (10 µg), Amikacin (30µg), Meropenem (10 µg), Tetracycline (30µg), Doxycycline (30 µg), Norfloxacin (10 µg), Nitrofurantoin (300 µg), Nalidix (30 µg), and Trimetho-Sulfamethazole (1.25+23.75 µg) using disc diffusion method in Muller-Hinton agar and interpreted according to [15].

3. Molecular identification of the most resistant *E. coli* isolate

The most resistant isolate was confirmed with molecular characteristics as 16S rRNA. PCR products were sequenced using the standard Sanger method on ABI 3730XL DNA Sequencer at MacroGen sequencing services (MacroGen, Seoul, South Korea). The partial nucleotide sequence of 16s rRNA region for bacterial isolate was done from the forward and reverse directions. All

molecular identification procedures were done in Omicsense Company (Al- Maady, Egypt).

4. Bacteriophage enrichment and isolation

Two sewage samples were collected from different locations and the enrichment technique was applied to isolate the *E. coli* phages according to Adam [16]. The crude lysate of the phages were obtained according to Borrego et al. [17] and assayed qualitatively by spot test and quantitatively the double-layer agar technique [16].

5. Phage purification and amplification

Bacteriophages were propagated and purified to ensure the purity of phage isolates using single plaque method to Adams [16].

6. Characterization of the isolated bacteriophages

6.1. Physical properties of bacteriophages

6.1.1. Thermal stability

Thermal inactivation point of phages *in vitro* was carried out by exposure *E. coli* phage to different degrees of temperature, 40, 50, 60, 60, 70, 80 and 90 °C for 10min using water bath and then immediately cooled under tap water. Treated phage was diluted and assayed by the plaque assay according to philipson [18].

6.1.2. pH stability

The ability of phage to survive at different pH levels was evaluated by exposing the phages suspension to different pH values from 2 to 12 using 0.1 M HCL/NaOH over 1 h at 37°C, the stability of survival was checked qualitatively and quantitatively by using plaque assay [19, 20].

6.1.3. Phages longevity *in vitro*

The infectivity of isolated phages was examined by plaque assay after incubation at different temperatures (4°C, room temperature and 20°C) for eight months [16].

6.2. Biological properties of bacteriophages

6.2.1. Host range of isolated bacteriophage

Host range of the isolated phages was determined using the spot test according to [21, 22, 23]. (The lytic spectrum of the isolated phages was determined against 26 *E. coli* isolates.

6.2.2. Phage adsorption rate

The adsorption rate of phages was determined by the method of Adams [16]. Phage suspension was added at a multiplicity of infection (MOI) of 0.1 to the host culture and incubated at 37°C. Aliquots were taken at 5 min intervals and the number of free infectious phage particles was calculated by phage titration.

7. Morphological properties of bacteriophages

In order to observe phage morphology, transmission electron microscopy (TEM) of the isolated five phages was performed as described by [24]. The examination was performed using a Hitachi H600A electron microscope at 80 KV at the Electron Microscopy Unit, Faculty of Agriculture, Mansoura University, Egypt.

Results

1. Isolation and identification *E. coli* and their antimicrobial susceptibility pattern

From collected diabetic foot samples, 27 bacterial isolates of *E. coli*(12.9%) were isolated. Bacterial isolates were gram negative, straight short rods, single, colonies are smooth, medium (2-3mm), shiny, circular pink red in color and lactose fermenters on MacConkey agar media (Fig. 1). Results of antimicrobial susceptibility pattern of *E. coli* in (Table 1) revealed that the highest percentage of resistance reached 88%to Ampicillin, Cefazolin and Norfloxacin, then 84% to Cefuroxime and Trimetho-Sulfamethazole1, 68% to Cefaperazone/ Sulbactam, Tetracycline, Aztreonam,72% to Carbenicillin, Cefotaxime and Cefepime, 92% to Ceftazidime, 60% to Amikacin, 64% to Nalidix and Ciprofloxacin, 48% to Tobramycin, 76% to Ofloxacin, 52% to Doxycycline, Chloramphenicol and 56% to Nitrofurantoin but it was sensitive 68% to Ceftriaxone 80% to 68% Gentamicin Polymixin B and colistin and 72% to Meropenem. The highly resistant bacterial isolate was chosen tightly for biochemical identification and for testing the susceptibility and MIC ($\mu\text{g/ml}$) with Vitek 2 (Table 2 & 3)and identified as *Escherichia coli*.



Figure 1. Photograph of *E. coli* on MacConkeyagar media

Table 1. The percentage of antibiotic resistance of *E. coli* isolates

Antibiotics	% of Resistance <i>E. coli</i> isolates
<i>Ampicillin</i>	85.2
<i>Carbenicillin</i>	63
<i>Cefaperazone Sulbactam</i>	66.6
<i>Aztreonam</i>	63
<i>Cefazolin</i>	85.2
<i>Cefuroxime</i>	81.5
<i>Cefotaxime</i>	70
<i>Ceftriaxone</i>	29.6
<i>Cefepime</i>	48.1
<i>Ceftazidime</i>	85.1

<i>Tobramycin</i>	44.4
<i>Amikacin</i>	55.5
<i>Gentamicin</i>	29.6
<i>Ciprofloxacin</i>	63
<i>Ofloxacin</i>	74
<i>Norfloxacin</i>	85.2
<i>Meropenem</i>	25.9
<i>Chloram- phenicol</i>	51.8
<i>Polymixin B</i>	18.5
<i>Colistin</i>	18.5
<i>Trimetho-Sulfamethazole1</i>	81.5
<i>Nalidix</i>	63
<i>Doxycycline</i>	48.1
<i>Tetracycline</i>	63
<i>Nitrofuration</i>	51.8

Table 2. Biochemical identification *E. coli* isolate by Vitek2 GP card

No. of tests	Biochemical tests	Result
1	Ala-Phe-Pro Arylamidase (APPA)	-*
2	L-Proline Arylamidase (ProA)	+*
3	Tyrosine Arylamidase (TyrA)	+
4	L-Lactate Alkalinization (lLATk)	+
5	O/129 Resistanec (Comp. Vibrio) O129R	+
6	D-Sorbitol (dSOR)	+
7	D-Mannitol (dMAN)	+
8	Alpha- Galactosidase (AGAL	-
9	Urease (URE)	-
10	Saccharose-Sucrose (SAC)	-
11	L- Pyrrolidonyl- Arylamidase (PyrA)	-
12	D- Maltose (dMAL)	+
13	Phosphatase (PHOS)	-
14	B-Glucoronidase (BGUR)	-
15	Lipase (LIP)	-
16	Tagatose (dTAG)	-
17	Alpha Glucosidase (AGLU)	-
18	Ornithine decarboxylase(ODC)	-
20	GlutamyleArylamidaseNA (AGLTP)	-
21	Palatinose (PLE)	-
22	D- trehalose (dTRE)	+

23	Succinate Alkanization (SUCT)	+
24	Lysine Decarboxylase (LDC)	+
25	D- Glucose (dGlucose)	+
26	d- Mannose (dMNE)	+
27	CIT citrate (sodium)	-
28	Beta N-Acetylc (NAGA)	-
29	L-Histidine assimilation (IHISa)	-
30	Gamma-Glutamyl-Transferase (GGT)	-
31	Beta- Xylosidase (BXYL)	-
32	Malonate (MNT)	-

Table 2, Continue

No. of test	Biochemical tests	Result
33	Alpha – Galactosidase (AGAL)	+
34	Beta Galactosidase (BGAL)	+
35	Fermentation/ Glucose (OFF)	+
36	Beta-Alanine Arylamidase PNA (BALap)	-
37	5-keto-D-Gluconate (5KG)	+
38	Hydrogen Sulphide (H ₂ S)	-
40	Beta-Glucosidase (BGLU)	-
41	Glycine Aryamidase (GlyA)	+

*+ = Positive and - = Negative.

Table 3. Susceptibility and MIC (µg /ml) for *E. coli* (D15c) isolate

D15c	Antibiotic group	Antimicrobial
+		<i>ESBL</i>
≥ 32(R)	B-lactams	<i>Ampicillin</i>
≥ 32(R)	B-lactams	<i>Ampicillin/ Sulbactam</i>
≥64(R)	Cephalosporin	<i>Cefazolin</i>

4(*R)	Cephalosporin	<i>Cefepime</i>
4 (*R)	Monobactams	<i>Aztreonam</i>
$\leq$ 0.5(S)	Carbapenems	<i>Ertapenem</i>
$\leq$ 0.25(S)	Carbapenems	<i>Meropenem</i>
$\leq$ (2S)	Aminoglycosides	<i>Amikacin</i>
$\geq$ (16R)	Aminoglycosides	<i>Gentamicin</i>
8 (I)	Aminoglycosides	<i>Tobramycin</i>
$\geq$ 4(R)	Quinolones	<i>Ciprofloxacin</i>
$\geq$ 8(R)	Quinolones	<i>Moxifloxacin</i>
64 (I)	Nitrofurans	<i>Nitrofurantoin</i>
0.55		<i>MDR Index</i>

S: Sensitive; I: Intermediate; R: Resistant;ESBL and Extended spectrum beta-lactamases

2. Molecular identification of multi- drug resistant *E. coli* isolates using 16S rRNA gene

16s rRNA region of multidrug bacterial isolates was amplified from isolated DNA of cells mixed with PCR reaction mixture and specific primer sets. The size of PCR product was estimated by comparing its electrophoresis mobility with those of standard DNA ladder (100 bps). The amplified DNA fragments were in the expected size calculated ~ 1300 bps(Fig. 2). The partial nucleotide sequence of 16s rRNA region for bacterial isolate was done from the forward (Fig. 3) and reverse direction and represented as 704bps for forward direction and 691 bps for reverse direction (Fig. 4). The phylogenetic tree was performed and showed that Egyptian isolate were be found to be highly homologous with *Escherichia coli* strain EGI255 16S ribosomal RNA gene that recorded on Gene-Bank and significant similarities for 16sRNA gene of *E. coli* isolate with some other related genera recorded in gene bank was done using

Blast research alignment (Table 4). The Gene-Bank nucleotide sequence accession numbers for partial sequences of 16S rRNA gene generated in this study using DDBJ Center and recorded as *Escherichia coli* EcEG01 LC589615.

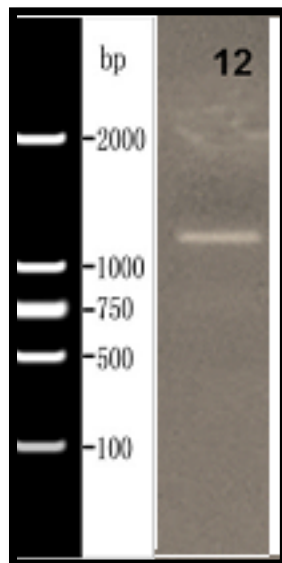


Figure 2. Agrose gel electrophoresis of polymerase chain reaction (PCR) of MDR *E. coli* isolate genes

Figure
Partial

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<H200410-014_I06_12_16S-F.ab1 704
ACGTATTGGATCGACTGCGATGCAGCCCTCCCGAAGGTTAAGCT
AACTACTTCTTTTGCTCCCACTCGCATGGTGGGACAGGCGGTGTG
TACAAGGCCCGGGAACGAATTCGCCGTGTAATTCTGATCCACGA
TTACAACCGATTCCGACTTCATGGAGTCAAGTTGCAGACTCCAA
TCCGGACTACGACGATGTTTATGAGGTCCGCTTGCTCTCGCGAG
GTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTG
GTCCCAAGGGCCCTGATGACTTGACGCCGTCCCCACCTTCCTCCG
AGGTATCACTGGGGAATTCCTTTGAGTGCCCGGCCGGACCGCTC
GCACCTGACGATAGGGGTAGCACTGGTTGCGGGACTTAACCCAA
CATTTCAACACGAACGACGACAGCCATGCAGCACCTGTCTC
ACAGTTCCCGAAGGCACAATTCCATCTCTGAAAAGTTCCGTGGA
TGTCAGACAGGTAAGTTCTTCGCGTTGCATCAAATTAAGCGC
CATGCTCCACCGCTTGTGCGGGCCCCCGTCAAATCATTGAGTTT
TAACCCTGCGGCCGAACCTCCTCTGAGACTGACTTAACGCATTA
TCTCCGAGAGGCCACACCTCTCCGGGACAACCTCCAAGTTGACA
TCATATACGGAGTGGACTACCAGGGTATCTAATCCTGCT
```

3.

nucleotide sequence of 16s rRNA gene for *E. coli*(forward direction)

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<H200410-014_C08_12_16S-R.ab1 691
GCGTGACGACTGACTGCGATGCAAGCCGTCCCGAAGGATGCGCTAA
CTACTTCTTTTCGCTCCCAATCGCCTGGCGGGAGAGTAATGTCTGGCA
AAGTGCCTGATGGAAGTCGATGACTACTGCTAATCCACGATTACAAC
GGATTCCGACTTCATACCAGTCAAGGGGACCACTCCGGTCTCGACTA
CTACGATGTTTATGATGGGATTTTGCTCTCGCGAGGTCTGCTTCTCTTT
GTATGCGCCATATCCCTACGTGTGTGACCTGGGACCAAGGGCCCTGA
TGATTTGACGCCGTCCCCACCTTCTCCGAGGTATCACTGGGGAATT
CCTTTGAATGCCCGGCCGGACCATTCGCACCTGACGATAGGGGTAGC
ACTGCCTGCGGGACTTAACCCACTTTTTCACAACACGAACTGACGAC
AGCCATGCCTTTCTGTCTCACCGTTCCCCGAAGGCACAATTCCATCTC
TGAAAAGTTCCGTGGATGTCAAGACCACGTAAGGTTCTTCGCGTTGC
ATCAAATTAAGCCACATGCTCCACCGCTTGTGCGGTCCCCCGTCAGA
TCATTTGAGTTTTAACTCTGCCTCCGAACTCCCCCTGATACTGACTTA
ACTCATTATCTCCGGAAGCCACACCTCTCCGGCACAACCTCCGAGTC
GACATCATTTACGGCGTGAACCTACCAGGGTA
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Figure 4. Partial nucleotide sequence of 16s rRNA gene for *E. coli*(reverse direction)

Table 4. Significant similarities for 16sRNA gene of *E. coli* isolate with some other related genera recorded in gene bank

Description	Query cover	Per. Ident.	Accession
<i>Escherichia coli</i> strain N12-3 16S ribosomal RNA gene, partial sequence	96	89.85 %	MT192513.1
<i>Escherichia coli</i> strain N11-4 16S ribosomal RNA gene, partial sequence	96	89.85 %	MT192512.1
<i>Escherichia coli</i> strain EC 16S ribosomal RNA gene, partial sequence	96	89.87 %	MN083306.1
<i>Escherichia coli</i> strain 73 16S ribosomal RNA gene, partial sequence	96	89.88 %	MH671439.1
<i>Escherichia coli</i> strain 56 16S ribosomal RNA gene, partial sequence	96	89.87 %	MH671432.1

3. Isolation, propagation of coliphages
Incidence of phages specific for clinical

bacterial isolates were done using spot test and confirmed by plaque assay technique. Results showed that four phage isolates were attacked *E. coli* and designated as C1, C2, C3 and C4.

4. Morphological characteristics of coliphages

4.1.1. Plaque morphology

Four isolated phages formed distinct plaques which differ in size and transparency. C1 phage formed plaque of medium size (3mm), irregular; C2 phage formed plaque of medium size 2mm, round and clear, clear plaque with halo, C3 phage formed a medium sized (3mm) irregular and clear plaque with halo and C4 phage formed plaque of medium size (2mm) round and clear. Two phages with different plaques morphology (C1, C2,) were selected for further characterization (Fig.5).

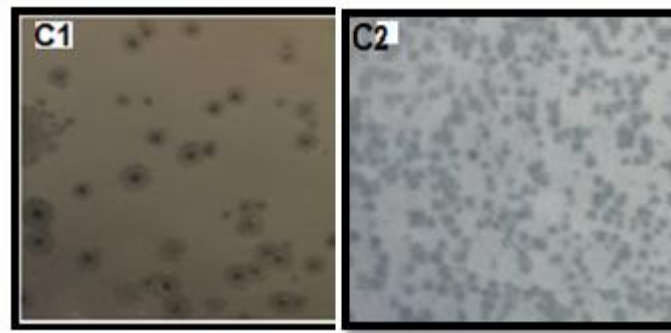


Figure 5. Photograph of plaque assay technique showing the morphological characters for C1 and C2 phages.

4.2. Morphological properties of phages (TEM)

Transmission Electron microscopy (TEM) revealed that both phage particles had head and long contractile tail. According to International Committee on Taxonomy of viruses (ICTV) the bacteriophages C1 and C2 resemble those of Caudovirales order. C1 phage contains hexagonal head with diameter about 96 nm and long tail with length about 120nm with diameter about 24 nm while, C2 phage contains elongated head with diameter about 78.5 nm and long tail with length about 135.7nm with diameter about 35.7 nm. Both phages assumed to belong to myoviridae family (Fig. 6).

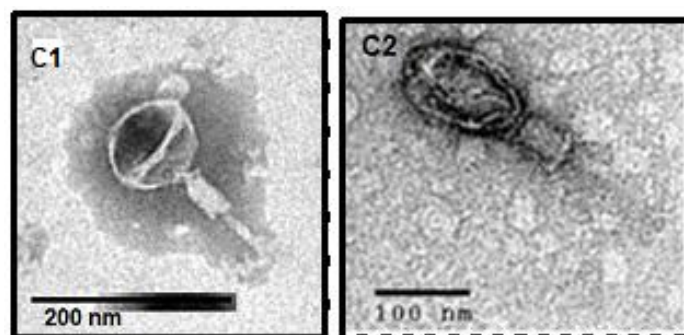


Figure 6. Electron micrograph illustrates bacteriophages C1 and C2 specific for *E.coli*.

5. Characterization of the isolated bacteriophages

5.1. Thermal Stability and pH Stability of the coliphages

From the obtained results, it was found that both phage particles remain infective from 40°C to 70°C and completely stopped at 80°C (Fig. 7). The results also revealed that the viral infectivity increased by increasing the pH value until it reach the maximum infectivity then decreased

gradually and lost their ability to lyse host at pH12. The maximum infectivity of both phages was in neutral medium at (pH=7). Both phages were lost their infectivity at pH 2 and 12. C1 phage still infective at pH ranged from 3 to11, while C2phage was lost their infectivity at 3 and 12. The result indicate that all phages were stable at pH 4-11, but inactivated completely at pH2 and 12 (Fig.8).

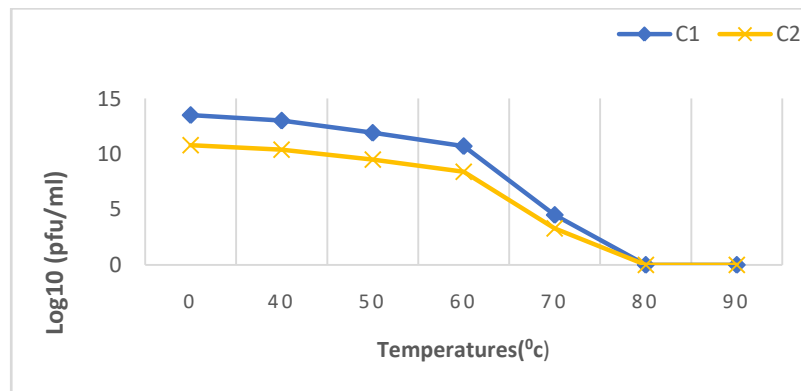


Figure 7. Stability of C1 and C2 phages at different temperatures.

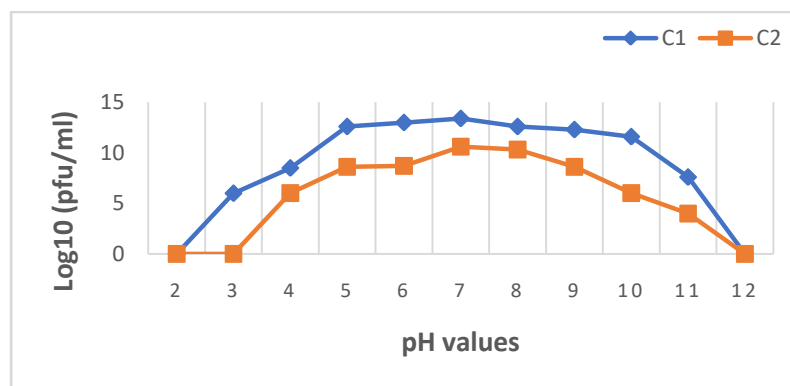


Figure 8. Stability of C1 and C2 phages under different pH values.

5.2. Determining the host specificity of C1 and C2 phages

Coliphages C1 and C2 showed variability in the lytic spectrum to clinical bacterial *E. coli* isolates. It was found that 15 bacterial isolates had sensitivity to C2 while, 22 bacterial isolates had sensitivity to C1 phage (Table 5).

Table 5. Host range pattern for C1 and C2 phages specific for *E. coli* isolates

Tested bacterial isolates	Phage isolates	
	C1	C2
1	-	-

2	+	+
3	-	+
4	+	+
5	-	-
6	+	-
7	-	+
8	+	-
9	+	+
10	+	+
11	+	-
12	+	+
13	+	+
14	+	-
15	+	+
16	+	+
17	+	+
18	+	+
19	+	-
20	+	-
21	+	+
22	+	+
23	+	+
24	+	-
25	+	-
26	+	-

+ = lysis & - = No lysis

5.3. Phages longevity and rate of adsorption

The infectivity of C1 and C2 phages was determined at month intervals. The results (Fig. 9) revealed that C1 and C2 phages were remain survive for eight months at 4°C with titer 0.1×10^6 and 0.1×10^5 respectively while, at room temperature C1 phage remain survive for 7 months with low titer of about 0.1×10^3 and C2 phage still infective for five months with low titer about 2×10^3 (Fig. 10). On the other hand, C1phage after stored at freezing (-20°C) was survived for five

months with low titer of about 0.9×10^3 PFU/ml and C2 phage survived for only four months with low titer about 1×10^3 (Fig. 11). The results in showed that the adsorption rate of C1 and C2 phage specific for *E. coli* was found to be 15 min.

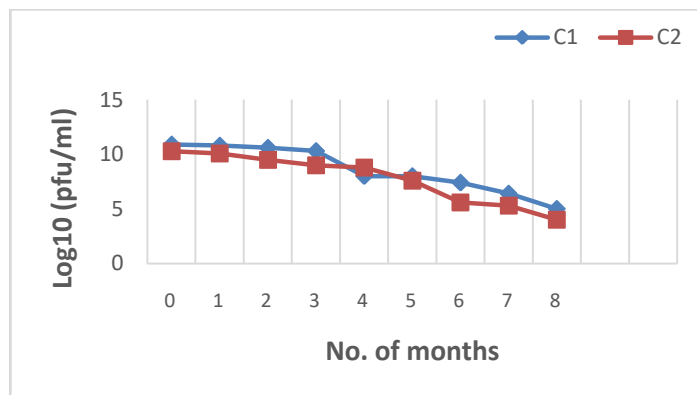


Figure 9.Determination the longevity of C1 and C2 phages at 4°C

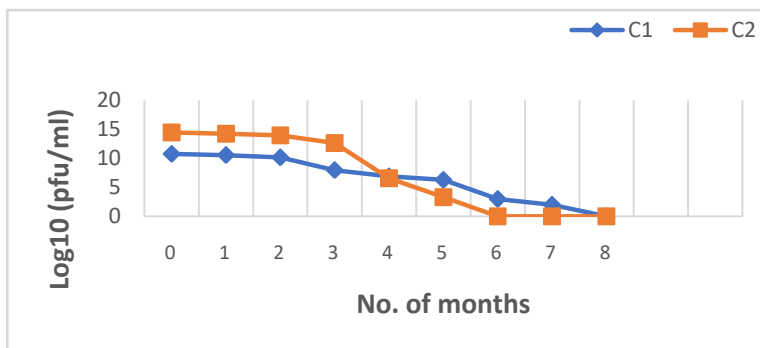


Figure 10.Determination the longevity of C1 and C2 phages at room temperature

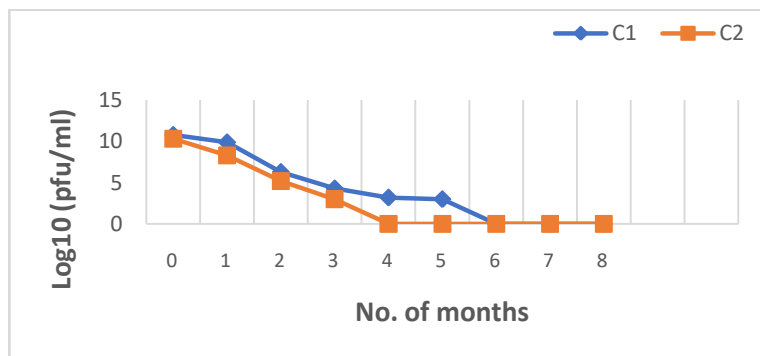


Figure11. Determination the longevity of C1 and C2 phages at -20°C

Discussions

Foot ulcers are considered the main cause of hospitalization and mortality of diabetic patients across the world. In recent years, the development of antibiotic resistant bacteria has made it increasingly difficult to select appropriate antibiotics for the treatment of DFI resulting in a significant upturn of morbidity and mortality [25, 26]. In the present study, the prevalence of *Escherichia coli* isolates in diabetic foot samples was 12.9%. The antibiotic resistance patterns of *E. coli* isolates were studied and the isolates were multi-drug resistant with percentage of 92%.

The present study revealed that *E. coli* showed the highest resistance to ampicillin, cefazolin and Norfloxacin, followed by Cefuroxime and Trimetho-Sulfamethazole¹, then to Cefaperazone/ Sulbactam, Tetracycline, Aztreonam. On the other hand, the isolates were sensitive to Ceftriaxone, Gentamicin, Polymixin B, colistin and Meropenem. These results were in compatible with Shanmugam et al. [27]; PHE [28] and Mutonga et al.[29].

In the present study, the partial genome sequence of *E. coli* was about 704bps for forward direction and 691 bps for reverse direction. These results were in agreement with Olowe et al. [30] who identified nine *E. coli* isolates using 16S rRNA gene sequence and found isolates were 91% similar to both *E. coli* strain.

In the current study four Coliphages were obtained from sewage samples and formed round or irregular plaques of medium size ranged between 2-3mm, and with or without halos. These results were in compatible with Necel et al. [31] who isolated vB_Eco4M-7 and ECML-117 phages that form uniform plaques on *E. coli* O157:H7 (ST2-8624) strain with diameters of approximately 1 mm.

The results of thermal stability for coliphage revealed that both phages specific for *E. coli* C1 and C2 remain infective at 70°C but inactivated completely at 80°C. In the same line, Litt & Jaroni [32] reported that the thermal stability studies on all the tested seven phages specific for *E. coli* O157 were stable in the 40–60°C temperature range and phage infectivity was affected at higher temperatures of 70 and 90°C. Furthermore, the pH stability of four coliphages were showed that phages C2 was lost their infectivity at 3 and 12 while, C1 phage still infective at pH. These results are in agreement with Manohar et al. [33] who reported that *E. coli* phages were infective from pH 4 - 11, but the phages were inactivated at pH 3 and 12. On the other hand, Litt & Jaroni [32] reported that the isolated phages specific for *E. coli* O157 were stable at a wide pH range (1–11). At low pH, it is possible for phages to acquire non reversible mutations [34, 35].

Host range pattern of coliphages C1 and C2 in the current study showed variability in the lytic spectrum to clinical bacterial *E. coli* isolates. 81.5% of *E. coli* isolates had sensitivity to C1 but, C2 infect only 55% of *E. coli* isolates. These results were incompatible with Manohar et al. [33] who reported that 73% of the tested *E. coli* isolates were sensitive to myPSH2311*Escherichia* phage using spot test.

Phage stability is an important aspect for ensuring phages activity during storage and transportation after preparation. Although phages may not necessarily be stored at 37 °C, storage conditions are not always constant especially in hot climates where refrigeration is not available. Type of phage and solute available in a sample may also effect on stability of phage [36].

In this study, the infectivity of C2 and C3 phages was determined at month intervals. The results revealed that C1 and C2 phages were remain survive for eight months at 4°C with percentage of 46% and 39%, respectively while, at room temperature C1 phage remain survive for 7 months with low percentage of 18.5% and C2 phage still infective for five months with percentage of 22.7%. On the other hand, C1 phage after stored at freezing (-20°C)

was survived for five months with percentage of 18.5% PFU/ml and C2 phage survived for only four months with low titer about 29.1%. Similarly, Litt & Jaroni [32] tested phages specific for *Escherichia coli* O157 for cold storage stability and found to be more stable at refrigeration temperature (4°C) than at frozen temperatures (−20°C and −80°C) over a period of 90 d. Previous studies have showed that tailed phages are more resistant to refrigeration and can remain viable for more than 10–12 years at 4°C [37].

The results showed that the rate of adsorption of C1 and C2 phage specific for *E. coli* was found to be 15 min. The transmission electron microscopy of coliphages revealed that C1 and C2 belong to Myoviridae family and contain head with diameters about 78.5 nm and 96 nm and long tail with length about 135.7 nm and 120 nm with diameters about 35.7 nm and 24 nm, respectively. These results were in agreement with Necel et al. [31] who isolated two phages of Myoviridae family. Both phages contain head of 66 nm in diameter and a contractile tail with diameter of 107 × 20 nm and 120 × 20 nm.

Conclusion

In order to overcome the appearance of multidrug-resistant bacteria, particularly human pathogens especially in diabetic foot infections, phage therapy was studied. Twenty seven *E. coli* isolates were obtained from diabetic foot patients and the most resistant one was confirmed with molecular analysis using 16srRNA gene. Specific phages for MDR *E. coli* isolate were isolated from sewage, identified and characterized. The bacteriophages were belonging to the family Myoviridae. They had wide host ranges, wide range of temperature, pH suggest that they can be used in further studies on investigation the effectiveness of bacteriophage against multi-drug resistant bacteria of DFU in animal model.

Abbreviations

DM: Diabetes mellitus; MDR: Multidrug resistant; DFU; Diabetic foot ulcer; PT: Phagetherapy; AMR: antimicrobial resistance TEM; Transmission Electron Microscope; MIC: Minimum inhibitory concentration; 16srRNA: 16S ribosomal RNA; PCR; Polymerase chain reaction; bps; base pairs; Pfu: Plaque forming unit; MOI: Multiplicity of infection.

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References

- [1] International Diabetes Federation (IDF) (2015). annual report.
- [2] Madacki-Todorović, K., Eminović, I., Mehmedinović, N. I., & Ibrišimović, M. (2018). Insulin Acts as Stimulatory Agent in Diabetes-Related *Escherichia Coli* Pathogenesis. *International Journal of Diabetes and Clinical Research*, 5, 098.

- [3] Tetz, G., Brown, S.M., Hao, Y. & Tetz, V. (2019). Type 1 Diabetes: an Association between Autoimmunity, the Dynamics of Gut Amyloidproducing *E. coli* and their Phages. *Scientific Reports*, 9, 9685.
- [4] Kostic, A. D., Gevers, D., Siljander, H., Vatanen, T., Hyötyläinen, T., Hämäläinen, A-M, Peet, A., Tillmann, V., Pöhö, P., Mattila, I., Lähdesmäki, H., Franzosa, E. A., Vaarala, O., Goffau, M., Harmsen, H., Ilonen, J., Virtanen, S.M., Clish, C.B., Orešič, M., Huttenhower, C. and Knip, M. & Xavier, R. J. (2015). The Dynamics of the Human Infant Gut Microbiome in Development and in Progression towards Type 1 Diabetes. *Cell Host Microbe*, 17(2), 260–273.
- [5] Gould, D. (2010). Causes, prevention and treatment of *Escherichia coli* infections. *Nurs Stand*, 24(31), 50-56.
- [6] Madappa, T. (2014). *Escherichia coli* infections. <http://tinyurl.com/qfxuocb> (accessed 29 January 2015).
- [7] Makvana, S. & Krilov, L.R.(2015). *Escherichia coli* Infections.*Pediatrics in Review*, 36 (4), 167-171.
- [8] Vivas, R., Barbosa, A. A. T., Dolabela, S. S. & Jain, S. (2019). Multidrug-Resistant Bacteria and Alternative Methods to Control Them: An Overview. *Microbial Drug Resistance*, 25(6), 890-908.
- [9] Bolocan, A.S., Callanan, J., Forde, A., Ross, P. & Hill, C. (2016). Phage therapy targeting *Escherichia coli* a story with no end? *FEMS Microbiology Letters*, 3639 (22).
- [10] Roach, D. R. & Debarbieux, L. (2017). Phage therapy: awakening a sleeping giant. *Emerging Topics in Life Sciences*, 1(1), 93–103.
- [11] Mendes, J. J, Leandro, C., Mottola, C., Barbosa, R., Filipa, A. S., Oliveira, M., Vilela, C. L., Melo-Cristino, J., Górski, A., Pimentel, M., São-José, C., Cavaco-Silva, P. & Garcia, M. (2014). In vitro design of a novel lytic bacteriophage cocktail with therapeutic potential against organisms causing diabetic foot infections. *Journal of Medical Microbiology*, 63(8), 1055-1065.
- [12] Parasion, S., Kwiątek, M., Gryko, R., Mizak, L., Malm, A. (2014). Bacteriophages as an alternative strategy for fighting biofilm development. *Polish Journal of Microbiology*, 63, 137–145.
- [13] Schmelcher, M. & Loessner, M.J., (2014). Application of bacteriophages for detection of foodborne pathogens. *Bacteriophage*, 4, e28137.
- [14] Akhi, M.T., Shirinzadeh, M., Ghotaslou, R., Sorous, M. H, Pirzadeh, T. & Behzad, M. N. (2013). Determination of antibiotic sensitivity of *bacteroid fragilis* isolated from patients and healthy individuals in Imam Reza Center of Medical Teaching and Treatment Tabriz. *Jundishapur Journal of Microbiology*, 6 (9).
- [15] Clinical and Laboratory Standards Institute (CLSI) (2018). *Performance standards for antimicrobial susceptibility testing*.
- [16] Adams, M.H. (1959). Bactriophages interscience Publishers, Inc., New York, 74, 164.
- [17] Borrego, J.J., Morifiigo, M. A., deVicente Cornax, R. & Romero, P. (1987). Coliphages as an indicator of faecal pollution in water: Its relationship with indicator and pathogenic microorganisms. *Wat. Res.*, 21: 1473-1480.
- [18] Philipson, L., Albertson, P. & Frick, G. (1960). Purification and concentration of virus by aqueous polymer phase system. *Virology*, 11,553-571.
- [19] Dhar, B., Singh, B., Singh, R., Singh, R., Singh, V. & Srivastava, J. (1978). Isolation of a virus (RL1) infective on *Rhizobium leguminosurum*. *Archives of Microbiology*, 119, 263-267.

- [20]Jamalludeen, N., Johnson, P., Robert, F., Kropinski, A., Erika, J. & Gyles, C. (2007). Isolation and characterization of nine bacteriophages that lyse O149 enterotoxigenic *E. coli*. *Veterinary Microbiology*, 124, 47-57.
- [21]Chopin, M., Chopin, A. & Roux, C. (1976). Defenition of bacteriophage groups according to their lytic action on mesophilic lactic *Streptococci*. *Applied andEnvironmental Microbiology*, 32, 741-746.
- [22]Goodridge, L., Gallaccio, A. & Griffiths, M.W. (2003). Morphological, host range and genetic characterization of two coliphages. *Applied Environmental Microbiology*, 69: 5364-5371.
- [23]Carlson, K. (2005). Working with bacteriophages: common techniques and methodological approaches. In: Bacteriophages: biology and applications. Kutter, E. and Sulakvelidze, A. (Eds.). CRC Press, Boca Raton, FL, 129-163.
- [24]Bradley, D.E. (1967). Ultrastructure of bacteriophage and bacteriocins. *Bacteriological reviews*, 31(4), 230.
- [25]Lipsky, B. A. (2016). Diabetic foot infection. Current treatment and delaying the Post – antibiotic era. *Diabetes Metabolism Research and Reviews*, 32 (1), 246- 2453.
- [26]Maciejewska, B., Olszak, T. & Drulis-Kawa, Z. (2018). Applications of bacteriophages versus phage enzymes to combat and cure bacterial infections: an ambitious and also a realistic application? *Applied Microbiology and Biotechnology*, 102(6), 2563-2581.
- [27]Shanmugam, P., Jeya, M. & Linda, S.S. (2013). The Bacteriology of Diabetic Foot Ulcers, with a Special Reference to Multidrug Resistant Strains. *Journal of Clinical and Diagnostic Research*, 7(3), 441-445.
- [28]Public health England (PHE) (2014). English surveillance programme for antimicrobial utilization and resistance (ESPAUR).1-143.
- [29] Mutonga, D.M., Mureithi, M.W., Ngugi, N.N. & Otieno, F.C.F. (2019). Bacterial isolation and antibiotic susceptibility from diabetic foot ulcers in Kenya using microbiological tests and comparison with RT-PCR in detection of *S. aureus* and MRSA. *BMC Research Notes*, 12, 244.
- [30]Olowe, B.M., Oluyeye, J.O., Famurewa, O., Ogunniran, A.O. & Adelegan, O. (2017). Molecular Identification of *Escherichia coli*& New Emerging Enteropathogen, *Escherichia fergusonii*, From Drinking Water Sources in Ado-Ekiti, Ekiti State ,*Nigeria Journal of Microbiology Research*, 7(3), 45-54.
- [31] Necel,A., Bloch, A., Nejman-Faleńczyk, S., Grabski, B., Topka, M., Dydecka, G., Kosznik-Kwaśnicka,K., Grabowski, Ł., Jurczak-Kurek,A., Wołkowicz T., Węgrzyn G. & Węgrzyn, A. (2020). Characterization of a bacteriophage, vB_Eco4M-7, that effectively infects many *Escherichia coli* O157 strains. *Scientific Reports*, 10, 3743.
- [32]Litt, K. & Jaroni, D. (2017). Isolation and Physiomorphological Characterization of *Escherichia coli* O157:H7-Infecting Bacteriophages Recovered from Beef Cattle Operations Pushpinder. *International Journal of Microbiology*, 7013236.
- [33]Manohar, P., Tamhankar, A. J., Lundborg, C.S. & Nachimuthu, R. (2019). Therapeutic Characterization and Efficacy of Bacteriophage Cocktails Infecting *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter* Species. *Frontiers in Microbiology*, 10, 574.
- [34]Jończyk, E., Kłak, M., Międzybrodzki, R. & Górski, A. (2011). “The influence of external factors on bacteriophages—review.” *Folia Microbiologica*, 56(3), 191–200.
- [35]Nobrega, F.L., Costa, A.R., Santos, J.F., Siliakus,M.F., Lent, J.W.M., Kengen,S.W.M., Azeredo, J. & Kluskens,L.D. (2016). Genetically manipulated phages with

improved pH resistance for oral administration in veterinary medicine.” *Scientific Reports*, 6, 39235.

- [36]González -Mene'ndez, E., Ferna'ndez, L., Gutie'rrez, D., Rodri'guez, A., Marti'nez, B. & Garcí'a, P. (2018). Comparative analysis of different preservation techniques for the storage of Staphylococcus phages aimed for the industrial development of phage-based antimicrobial products. *PLoS ONE*, 13(10), e0205728.
- [37]Vasan, A., Leong, W. M., Ingham, S. C. & Ingham, B. H. (2013). Thermal tolerance characteristics of non-O157 Shiga toxigenic strains of *Escherichia coli* (STEC) in a beef broth model system are similar to those of O157:H7 STEC. *Journal of Food Protection*, 76, 1120–1128.