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Isolation And Characterization Of Some Bacteriophages Infecting Multi Drug Resistant Bacteria Isolated From Diabetic Foot Ulcers

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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. In the current study, 32 infected diabetic foot ulcer patients in sharkia Governorate (ElAhrar hospital in Zagazig, Sharkia, Egypt and private diabetic foot center in Fakous, Sharkia, Egypt) 64 bacterial isolates were collected during the period of October 2018 to January 2019.Resistance to antibiotics reached to 75% of these isolates .isolate no 8,25,28,29 and 30 gave the highest resistant to antibiotics. These isolates identified as *(Klebsiella pneumonia, proteus mirabilis, Providencia rettergi, Shigella sonnei, and E.coli)* .three bacterial viruses active against these bacteria were isolated .these phages named as ØSS29, ØKP8, and ØEC30.
As ØEC30 virus active against *E.coli,* active against ØKP8 *Klebsiella pneumonia* and ØSS29 *active against Shigella sonnei.*. The present study opens a new window for the application of bacteriophages as promising antimicrobial agents against MDR bacteria.

Keywords : Isolation, Characterization, Bacteriophage, Resistant bacteria.

I. Introduction

The world is facing a major epidemic of diabetes mellitus (DM).The global diabetes prevalence in 2019 is estimated to be 9.3% (463 million people), rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045 [1].All DM patients are at risk of developing a diabetic foot ulcer (DFU), which is a full-thickness wound below the ankle, irrespective of duration [3]. Even with the best preventive care, 9% of patients will develop a diabetic foot infection (DFI) [3].Patients with DM frequently require minor or major amputations of the lower limbs (15 to 27%), and in more than 50% of cases, infection is the preponderant factor [4].Major amputation is associated with significant morbidity and mortality (ranging from 13 to 40% at 1 year to 39 to 80% at 5 years, in addition to immense social, psychological and financial consequences [5]. Antibiotic treatment of infected ulcers is complicated by formation of microbial biofilms, which are often heterogeneous and resistant to antibiotics [6]. The rapid rise of multi-drug resistant bacteria worldwide has led to a renewed interest in phage therapy as a possible alternative to antibiotics or, at least, a supplementary approach for the treatment of some bacterial infections. Recently, the results of bacteriophage and phage cocktail application for the treatment of various infections have been reported in a number of clinical cases, case series and clinical trials [5].Bacteriophages are small viral entities, existing as nucleic acids packaged within a protein capsid, that specifically infect bacteria. Depending on their nature, after injection of their nucleic acids inside the bacteria, bacteriophages can either reside as a stable element called prophage inside the host cell as a free plasmid molecule or integrated into the host chromosome (temperate bacteriophages), or induce lysis of the bacterial host with the release of newly formed viral particles (lytic bacteriophages) [8].Bacteriophage therapy is widely used and generally accepted as safe and beneficial in some parts of the world [9].and recent trials in animal models have demonstrated their potential to improve or heal bacterial skin infections following both internal and external application [11,12]However, there is little experimental evidence demonstrating that bacteriophages can cure chronic infections established for more than several hours [13].
The aim of the current study was to isolate and recognize bacteriophages specific for some Multi drug resistant bacterial strains isolated from diabetic foot infected ulcers and investigating of their physical and biological properties.

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II. Material and methods

A.Isolation of multi drug resistant bacteria

This prospective study included 32 consecutive patients with diabetes and foot ulcers, who were admitted to ElAhrar hospital in Zagazig, Sharkia, Egypt and private diabetic foot center in Fakous, Sharkia, Egypt during the period of October 2018 to January 2019. The patients underwent extensive debridement of their diabetic foot ulcers, and all patients were taking antibiotics. Culture specimens were collected using sterile cotton swabs to eliminate the possibility of isolating colonizing bacteria. Swabbing was done on slouphy or inflammmed tissue as bacteria tend to present in greater number in these areas. Superficial ulcers were excluded from the study. After rinsing the wound area with saline and debriding the wound, samples were collected aseptically from the wound, conditioned in Stuart medium, and immediately taken to the microbiology laboratory. 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 using Gram staining technique and biochemical characteristics using Vitek2 system .

All isolates were tested for their susceptibility to different antimicrobial agents using Bauer disk diffusion method [15].

Isolates were tested for susceptibility to Cefuroime (30µg), Cefadroxil (30µg), Pencillin G (10µg), Amoxicillin/clavulanic acid (30µg), Imipenem (10µg), Polymixin B (300µg), Fusidic Acid (10µg), Amikacin (30µg), Erythromycin (15µg), Rifamycin (30µg), Levofloxacin (5µg), and Trimethoprim/sulphamethoxazole (25µg).

From the results of Disc diffusion method, most multi drug resistant bacterial isolates were selected and the antibiotic susceptibility profile was determined for these isolates by VITEK 2 automated system as following: an aliquot of 145µL of cellular suspension, with an optical density between 0.55 and 0.63, was added in 3mL saline solution (0.45% NaCL, pH 7.0) into a clear plast (polystyrene) test tube (12mm×75 mm). Samples were analyzed by VITEK 2 which indicated resistance or sensibility shown by the bacteria through several antibiotic concentrations in the anti-biogram cards.Susceptibility profiles were performed with 17 antibiotics, Ampicillin, Cefazolin, Ceftriaxone, Cefepime, Aztreonam, Ertapenem, Imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Ampicillin / Sulbactam, Moxifloxacin, Tigicycline, Nitrofurantion and Trimethoprim/Sulfamethoxazole were determined according to Clinical Laboratory Standard Institute (CLSI) guidelines, 2015 **.**

B. Identification of the most resistant isolates:

B.1. Identification of the most resistant isolates by VITEK-2 system: Identification was confirmed using API 20E strep strip systems (Biomerieux), according to manufacturer´s recommendations, VITEK method, according to manufacturer´s recommendations at Biotechnology unit, Animal Health Research Institute, Dokki, Giza, Egypt. VITEK-2 system imparts an automated, computer based technique of species identifications, relies on advanced colorimetry technology, the measurement of light attenuation associated with each biochemical reactions in VITEK cards The reagent cards have 64 wells and each well contain an individual test substrate. The 64-well plastic 2 GP Card cards contain 44 tests. Substrates assess various metabolic activities such as alkalinisation, acidification, enzyme hydrolysis, and growth in the presence of inhibitory compounds. The cards are inoculated with a 0.5 McFarland suspension of the organism prepared from an 18- to 20-h-TSA agar plate (bioMérieux) and are then automatically sealed and manually inserted inside the VITEK 2 reader-inoculator module. Fluorescence is measured every 15 min, and the results of identification are determined after 3 h.

B.2. PCR analysis for MDR isolates : DNA extraction from MDR bacterial isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. PCR amplification of 16S rRNA gene was performed according to [17].

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III. Isolation, Propagation and Purification of phages

Three Bacteriophages specific for multi-drug resistant (**Klebsiella pneumonia, Shigella sonnei**, **E.coli**) were isolated from sewage water samples obtained from Sharkia Governorate, Egypt by the enrichment technique [18].For this, the sewage sample was clarified by centrifugation at 6000 r.p.m for 20 min and the supernatant was filtered through a 0.45µm membrane filter.Then,1 ml of filtrate was mixed overnight with selected bacterial strains and incubated at 37 °C for 24h.About 10ml chloroform was added to the culture and bacteria debris wew removed by centrifugation at 6000Xg for 10 min. Ten microliters of the supernatant was spotted onto different bacterial strains including the strain used for phage enrichment. phages was recovered for lysis zones. phages were propagated and purified from singleplaque isolates according to [18].A single plaque was picked and put into a log phase culture of selected bacterial strains .After being incubated at 37 °C for 37 h, the phage host mixture was centrifuged ar 10,000 Xg for 10 min and filtered through a 0.45µm membrane filter followed by precipitation of the phage particles in the presence of 0.5 M NaCl and 5% polyethylene glycol 6000.
The pellet collected by centrifugation at 10,000 Xg for 30 min at 4 °C was dissolved in SM buffer (50Mm Tris Hcl at PH 7.5, 100 Mm NaCl,10 Mm MgSo4 and 0.01% gelatine). Purified phages were stored at 4 °C until used.
Nomenclature of isolated phages: The three phages specific for multi-drug resistant (Klebsiella pneumonia, Shigella sonnei, E.coli) designated as (ØKP8), (ØSS29), (ØEC30) respectively.

IV. Characterization of the isolated bacteriophages

4.1. Physical properties of isolated bacteriophages.

4.1.1. Thermal stability: Thermal inactivation point of phages *in vitro* was carried out by exposure phages to different degrees of temperature, 30,40, 50, 60, 60, 70, 80 and 90^{o} 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 [19].
4.1.2. PH stability: The ability of phage to survive at different pH levels was evaluated by exposing the phages suspension to different pH values from 3 to 11 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 [20,21].
4.1.3. Effect of U.V irradiation on the isolated phages: Phages lysate (diluted 0.1 in saline solution) were exposed directly to ultraviolet radiation 254nm, 70 µW/cm^2 at 15 cm distance from the UV source for 5, 15, 30, 45, 60, 75 and 90min, Then Plaque assay was performed.

4.2. Biological properties of bacteriophages:

4.2.1. Phages adsorption rate: The three phages exhibited different adsorption rates .These results showed that, The maximum adsorption rate for the different 3 phages was after (15) minutes but with different values As phage (**ØSS29**) had the highest value for maximum adsorption rate (74.2%) ,followed by phage (**ØKP8**) with maximum adsorption rate value (65.1%) and then in last phage (**ØEC30**) with maximum adsorption rate value (58.4%).
4.2.2. **One-step growth curve:** The latent and rise periods as well as burst size of each phage were determined using one-step growth curves. Result indicated that the latent periods for phages **ØSS29, ØKP8, ØEC30** were found to be 20,15 and 15 minutes, respectively .The burst size was phages per cell. Determination of burst size was based on the ratio of the mean yield of phage that infected the bacterial cells to the mean number of phage particles liberated.

V. Morphological properties of bacteriophages

In order to observe phage morphology, transmission electron microscopy (TEM) of the isolated three phages was performed as described by [22]. The examination was performed using a Hitachi H600A electron microscope at 80 KV at the Electron Microscopy Unit, Faculty of Agriculture, Mansoura University, Egypt.

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IV. Result

Table1A: Demographic and presence of bacterial isolates in 32 patients with DFI

parameters: Ages	(31:45) n=(4) (12.5%)				(46:60) n=(12) (37.5%)				(61:75) n=(16) (50%)				Total
Gender	♂		♀		♂		♀		♂		♀		32 (100%)
N	3 (75%)		1 (25%)		8 (66.66%)		4 (33.33%)		9 (56.25%)		7 (43.75%)		♂=20 (62.5%) ♀=12 (37.5%)
Growth on Medium total	MAC	B.A	MAC	B.A	MAC	B.A	MAC	B.A	MAC	B.A	MAC	B.A	–
	3	3	1	2	8	10	4	4	9	11	6	8	69
Gram(+)stain	0	2	0	0	1	4	2	3	0	5	0	3	20
Gram(–)stain	3	1	1	1	7	4	2	1	9	4	6	5	44
Total isolates	8 (12.5 %)				24 (37.5 %)				32 (50%)				64 (100%)

N = number, ♂= Male, ♀= Female, MAC= MacConkey Agar media, B.A= Blood Agar Media, (+) = Positive, (-)= Negative.

Table 1B:

patiant no.	Gender		Age	Growth on MAC&B.A		Antibiotic used in treatment	concentration
	♂	♀		MAC	B.A		
1	♂	–	61	+++	++	Ciprofar	CIP400mg
2	♂	–	56	++	+	Avelox	MXF400mg
3	♂	–	40	++	++	Avelox	MXF400mg
4	–	♀	55	+++	+	No antibiotic	–
5	–	♀	69	++	+	Ciprofar	CIP400mg
6	–	♀	67	+++	+	Augmentin	AMC1g
7	–	♀	55	++	+	Amikacin	AK500mg/2ml
8	–	♀	63	++++	+++	Amikacin	AK500mg/2ml
9	♂	–	50	++	++	Tavacin	LEV500mg
10	♂	–	48	+	+++	Augmentin	AMC1g
11	♂	–	56	+	++++	Curam	AMC1g
12	–	♀	58	+	+++	Tavacin	LEV500mg
13	♂	–	63	+	++	Amikacin	AK500mg/2ml
14		♀	70	++	+	Avelox	MXF400mg
15	♂	–	62	+++	+	No antibiotic	–
16	♂	–	64	++	++	Tavacin	LEV500mg
17	–	♀	67	++	++	Curam	AMC1g
18	♂	–	65	++	++	Curam	AMC1g
19	–	♀	66	++	+++	Ciprofar	CIP400mg
20	♂	–	43	+	+	Tavacin	LEV500mg

No: number/♂: Male /♀: Female/ MAC: MacConky media / B.A: Blood Agar/ (+): intensity of growth/CIP:Ciprofloxacin/MXF:Moxifloxacin/LEV:Levofloxacin/AK:Amicacin.

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Table (2): Antibiotic susceptibility of bacterial isolates by disc diffusion.

Antibiotic / Isolate Code	B-lactams					No. lactams Glycopeptide	Fusidanme	Protein synthesis		MRNA synthesis	Fluoro Quinolones	F.A synthesis Sulphonamide	MAR index	% of R
	CXM 30	CFR 30	P_1 0	Ams 30	IPM 10	PB_{300}	$F.A_{10}$	AK_3 0	E_1 5	$R.F_{30}$	$(Leo)_5$	$(SXt)_{25}$		
M1	15	0	30	0	26	20	10	18	0	12	0	17	0.33	33%
M2	0	9	22	18	25	18	10	15	15	0	8	11	0.16	16%
M3	0	18	10	20	19	7	17	12	18	0	0	0	0.33	33%
M4	12	12	13	8	28	12	6	18	8	8	9	15	0	0%
M5	7	17	9	0	26	28	7	7	0	7	17	7	0.16	16%
M6	0	13	20	7	27	6	8	27	12	0	0	15	0.25	25%
M7	9	10	15	0	20	0	0	16	9	7	8	16	0.25	25%
M8	0	0	13	0	22	0	0	15	0	0	0	14	0.66	66%
M9	0	12	8	0	18	0	8	7	18	7	0	15	0.33	33%
M10	0	0	18	0	29	0	0	18	0	0	0	12	0.6	60%
M11	6	10	30	0	25	24	7	20	19	0	0	19	0.25	25%
M12	0	0	30	0	30	15	10	25	11	13	0	15	0.33	33%
M13	0	0	25	0	30	0	11	23	0	0	0	15	0.5	50%
M14	0	0	35	0	32	25	15	23	0	0	0	18	0.5	50%
M15	0	0	32	0	30	0	13	25	0	0	0	15	0.58	58%
M16	0	0	25	0	30	0	12	23	0	0	0	0	0.66	66%
M17	10	0	30	15	30	25	12	25	13	0	0	15	0.25	25%
M18	0	0	33	0	32	12	12	23	0	0	0	15	0.5	50%
M19	0	0	34	0	32	15	12	23	0	0	0	15	0.5	50%
M20	0	0	30	0	30	0	12	25	0	0	0	13	0.5	50%
M21	0	0	35	0	35	15	12	23	0	0	0	18	0.5	50%
M22	0	0	30	0	28	25	12	25	15	0	0	20	0.41	41%
M23	0	12	35	17	36	35	12	22	20	15	0	20	0.16	16%
M24	0	0	25	0	30	0	0	22	0	0	0	0	0.75	75%
M25	0	0	30	0	30	0	10	25	0	0	0	15	0.58	58%
M26	0	0	30	0	20	20	0	32	0	0	0	20	0.58	58%
M27	0	0	30	0	27	25	12	21	0	0	0	15	0.5	50%
M28	12	0	27	0	30	0	17	15	0	0	0	25	0.5	50%
M29	0	0	28	0	35	0	0	22	0	0	0	16	0.66	66%
M30	0	0	27	0	18	0	0	18	0	0	0	15	0.66	66%
M31	0	0	40	0	50	15	0	25	20	0	0	16	0.5	50%
M32	0	18	35	18	50	30	20	25	20	0	0	18	0.25	25%

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Table3 .Biochemical identification of the most resistant isolates by Vitek2 GP card

NO. of test	BIOCHEMICAL TESTS:	Isolates no:				
		8	25	28	29	30
1	Ala-Phe-Pro Arylamidase(APPA)	-	-	-	-	-
2	H2S Production (H2S)	-	-	-	-	-
3	Beta-Glucosidase (BGLU)	+	-	+	-	-
4	L-Proline Arylamidase (ProA)	-	+	-	+	+
5	SACCHAROSE/Sucrose (SAC)	+	+	-	-	+
6	L-LACTATE alkanization (ILATK)	+	+	-	+	+
7	Glyeine ARYAMIDASE (GLYA)	+	-	-	+	-
8	(O/129 RESISTANCE Comp.vibrio) O129R	-	+	-	+	-
9	ADONITOL (ADO)	+	-	+	-	-
10	BETA - N -Acetyl-Glucamindase (BNAG)	-	-	-	-	-
11	D-MALTOSE (DMAL)	+	+	-	+	-
12	LIPASE (LIP)	-	-	-	-	-
13	TAGATOSE (DTAG)	-	-	-	-	-
14	(ALPHA -GLCOSIDASE) (AGLU)	-	+	-	-	-
15	ORNITHINE DECARBOXYLASE (ODC)	-	-	-	-	-
16	(GLU-GLY-ARG-ARYLAMIDASE) (GGAA)	-	-	-	-	-
17	L-Pyrrolydonyl-ARYLAMIDASE (PYRA)	+	-	-	-	-
18	Glutamyle Arylamidase PNA (AGLTP)	-	-	-	-	-
19	D-MANNITOL (dMAN)	+	-	+	+	+
20	PALATINOSE (ple)	+	+	-	-	-
21	D–TERHALOSE (d TRE)	+	-	-	+	+
22	SUCCINATE –alkanization (Suet)	+	+	+	+	+
23	LYSINE DECARBOXYLASE (LDC)	+	-	-	+	+
24	L–MALATE assimilation (IMLTa)	-	-	-	+	-
25	L–ARABITOL (IARL)	-	-	+	-	-
26	D–GLUCOSE (d GLU)	+	+	+	+	+
27	D–MANNOSE (d MNE)	+	-	+	+	+
28	TYROSINE ARYLAMIDASE (TyrA)	+	+	+	+	+
29	CITRATE SODIUM (CIT)	+	-	+	-	-
30	BETA A–N–ACETYL (NAGA)	-	-	-	-	-
31	L–HISIDINE assimilation (IHISa)	-	-	-	-	-
32	ELLMAN (ELLM)	-	+	+	-	-
33	D–CELLOBioSE (d CEL)	+	-	+	-	-
34	GAMMA–GLUTAMYLE–TRANSFERASE (GGT)	+	+	-	-	-
35	BETA–XYLOSIDASE (BXYL)	+	-	-	-	-
36	UREASE (URE)	+	+	-	-	-
37	MALONATE (MNT)	+	-	+	-	-
38	ALPHA–GLACTOSIDASE (AGAL)	+	-	-	+	+
39	CUMARATE (CMT)	-	+	-	+	+
40	L–LACTATE assimilation (ILATa)	-	-	+	+	-
41	beta–GLACTOSIDASE (BGAL)	+	-	-	+	+

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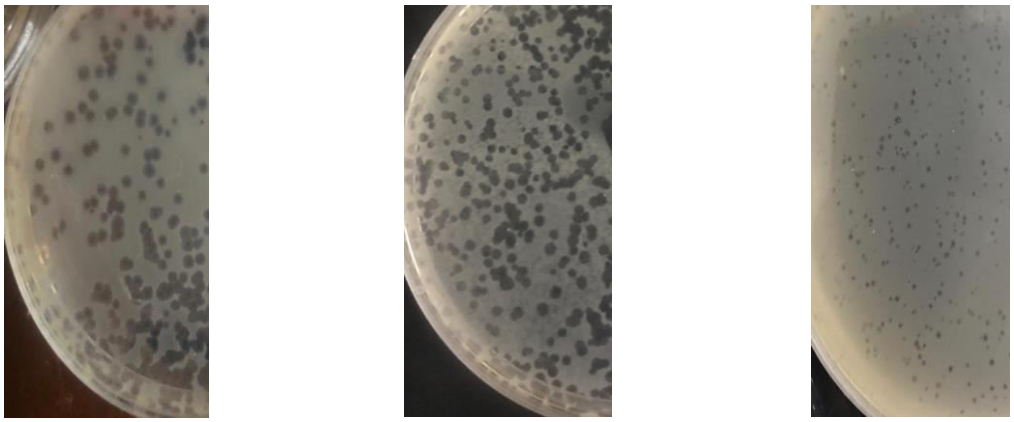
Plaque morphology: Three isolated phages formed distinct plaques which differ in size and transparency. **(ØKP8)** phage formed plaque of medium size (2mm) with clear center hale, **(ØSS29)** phage formed plaque of size (1mm), round and clear, phage (ØEC30) formed pen point. (P.P) size.



(ØKP8) **(ØSS29)** (ØEC30)

Figure 1: Photograph of plaque assay technique showing the morphological characters for **(ØKP8), (ØSS29)** and **(ØEC30)** phages**.**

Morphological properties of phages (TEM): Isolated phages (ØKP8), (ØSS29) ,(ØEC30) were viewed by Electron microscope **(Fig.)**.Phage **(ØKP8)** belongs to Podoviridae family and appeared as isometric head with very short non contractile tail. Phage **(ØSS29)** belongs to Myoviridae family and appeared as isometric head with short contractile tail.Phage **(ØEC30)**
Belongs to Siphoviridae family and appeared as isometric head with non.contractile tail.

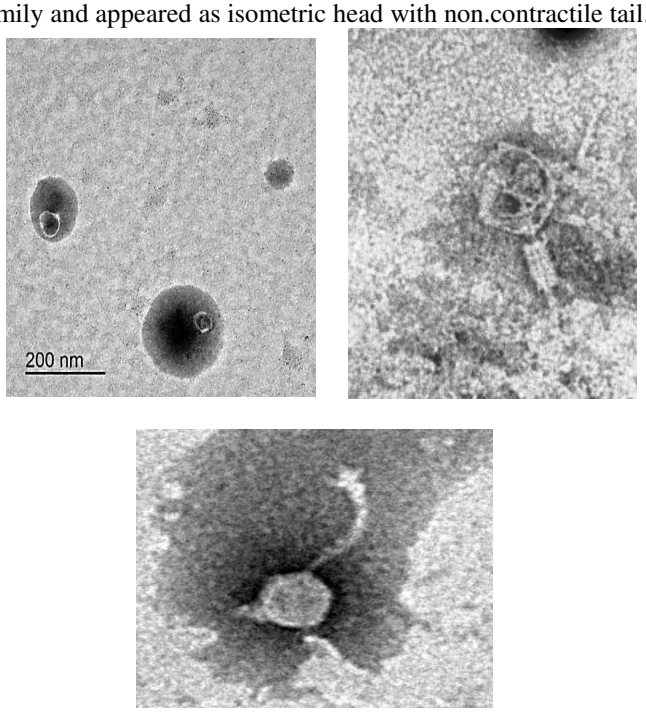


Figure 2: Electron micrograph illustrates bacteriophages (ØKP8), (ØSS29), (ØEC30) specific for *Klebsiella pneumonia, Shigella sonnei, Eschrichia coli* respectively.

Thermal stability: The stability of isolated phages at different temperatures was investigated. The results in (Fig3.) indicated that the ØEC30 phage was the most thermostable phage with the highest titer, as its titer was 2.8×10^{10} PFU/ml at 80 C°. Both ØSS29, ØKP8 phages was remain infective from 30 C° to 70 C° and completely stopped at 80°C.

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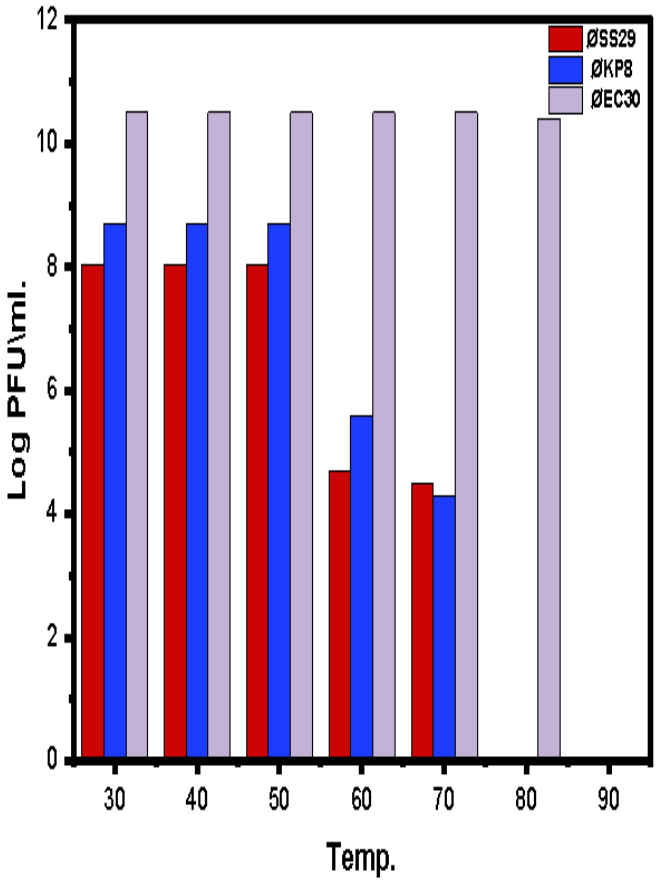

Figure 3: Thermal stability

PH stability: Data in **Figure(4)** demonstrated that The phages were able to survive over a wide pH range**.** For instance, phages survived pH range of 3 to 11 with maximum activity observed at pH

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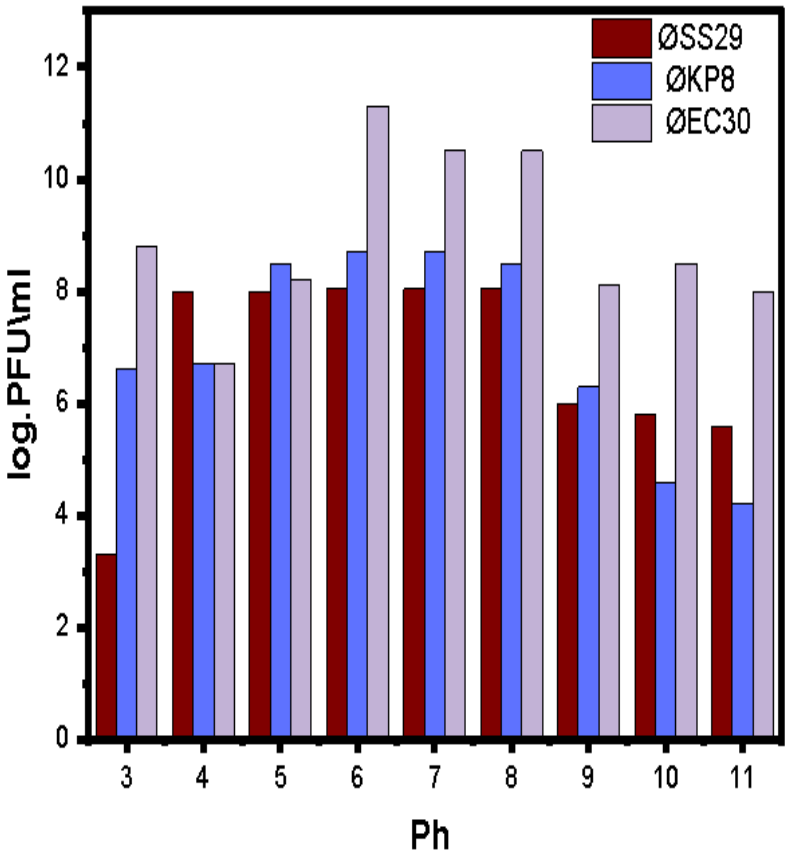

Figure 4: PH stability

Effect of U.V irradiation on the isolated phages: As shown in Figure (5), three *phages* didn't lose their infectivity after exposure to UV radiation at 15cm distance for 90min.

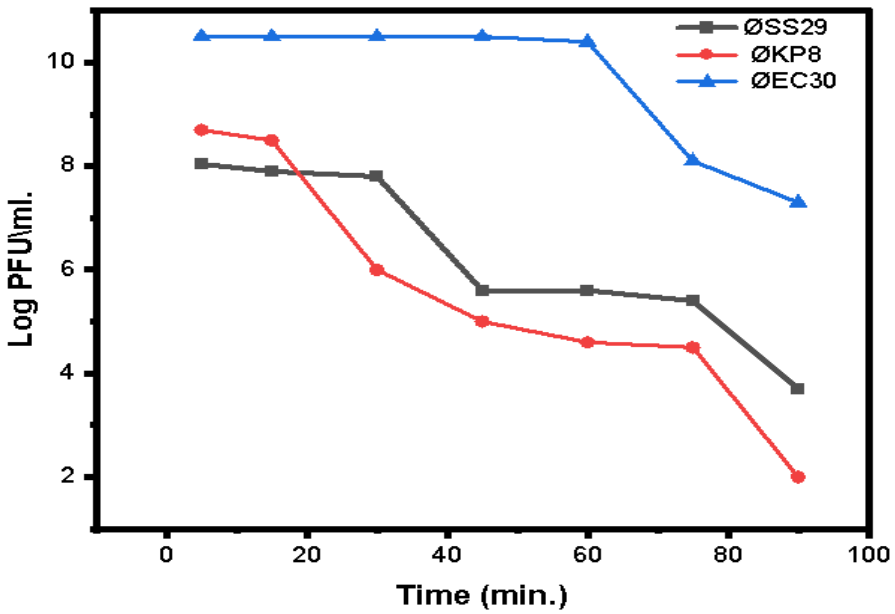

Figure 5 : Effect of U.V irradiation

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VI. CONCLUSION

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 . in order to overcome the appearance of multidrug-resistant bacteria, particularly human pathogens especially in diabetic foot infections, phage therapy was studied.

VII. Abbreviations

DM: Diabetes mellitus; **MDR**: Multidrug resistant; **DFU**; Diabetic foot ulcer; **PT**: Phage therapy; **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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