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Isolation of Lytic Bacteriophages to Bacteria Isolated from Infected Skin Burns

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Abstract

In this study, 30 bacterial isolates from burn patients were enrolled (10 isolates from each of *Acinetobacterbaumanii*, *Pseudomonas aeruginosa* and *Klebsiellapneumoniae*), along with various crude samples for phage isolation collected from different environmental sources in various regions in Baghdad from June 2020 to November 2020. Each bacterial isolate was subjected to a full set of diagnostic and antibiotic sensitivity tests by using the Vitek 2 compact system and Kirby-Bauer method; in addition, matching lytic phages to the diagnosed bacteria were isolated after tested byPhage spot lysis assay. The results showed that all bacterial isolates involved in this study were highly resistant to most antibiotics, and some of these isolates resistant to all antibiotics used in this study.Four lytic phages were isolated against *A. baumannii* isolates, also four lytic phages were isolated against *P. aeruginosa* isolates and three lytic phages were isolated against *K. pneumoniae* isolates from sewage, farm soil, wastewater, faeces of sheep and chicken litter. Finally, we proved the presence of lytic phages in the phage cocktail by using transmission electron microscopy.

Keywords: Infected skin burns, lytic bacteriophages

Introduction

Infection in the burn patient is the chief reason for morbidity and mortality. The source of bacteria in the burned patient could be either normal flora (endogenous) from the patient themself or exogenous from the environment or healthcare team. In general, exogenous organisms have a higher resistance than endogenous ones[1].Burn patients are very susceptible to infection due to the loss of the skin barrier, lengthened hospital stays, intensive invasive diagnostic and therapeutic procedures[2]. Therefore, normally 4-5 hours after the burn the wound surface becomes contaminated with various bacteria, which will start to grow and multiply, they will reach the vascular and lymphatic vessels and start to disperse. At this point, bacteremia and sepsis start[3]. Multi-drug resistance (MDR) bacteria like (*Pseudomonas aeruginosa, Acinetobacterbaumannii and Enterobacteriaceae*) is an emergent problem in burn patients, which occurs mainly due to prolonged courses of broad-spectrum antibiotics, all MDR bacteria may cause infections that make treating infections in burn patients a challenging problem because there are few antimicrobials still effective against them[4]. These times scientists are now challenging the threat of superbugs, i.e. pathogenic bacteria resistant to most or all available antibiotics which is a cause of major concern[5, 6]. Thus, investigating alternative approaches to develop antibacterial products is also an important duty, and re-examining the potential of promising older methods might be of benefit. One of the potential replacements for antibiotics is the use of bacteriophages virus or simply phages as antimicrobial agents[7, 8]. Phages are natural bacteria killers, proven as the best biocontrol agents due to their ability to lysis host bacterial cells specifically thereby helping in disease prevention and control[9].

Patient and method

A total of (42) different bacteria isolated (16 *Pseudomonas aeruginosa*, 14 *Klebsiellapneumoniae* and 12 *Acinetobacterbaumannii*), belonging to 38 patients (two patient have mix growth) with infected skin burnsin a different part of their body, however, only 30 isolates were enrolled in this study (10 from each genus) because of their highly multiple-drug resistance. The age of patients ranged from 1 year to 68 years and male to female ratio was 1:1.Bacterial Samples were collected in the Burn hospital of Medical City, Baghdad. Bacterial sampling was carried out during the period from November 2019 to March 2020.Identification of the isolates was depending on morphological characteristics, gram stain and biochemical tests and confirmation the results with Vitek 2 system.

Antibiotic susceptibility test

Antibiotic susceptibility test was carried out on bacterial isolates using Kirby-Bauer method[10].0.5 McFarland standards was used and the bacterial lawn was prepared by transferring enough growth from a pure culture into a

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tube of normal saline solution and spread bysterile cotton swab on Muller-Hinton agar Medium. Then, the inoculum was left to dry for a few minutes and the antibiotic discs were put on the inoculated plates with the aid of sterile forceps. The plates were left in an incubator upside down at 37°C for 18-24h.The antibiotic disks used in this study were Amikacin (30µg), Imipenem (10μg), Gentamicin (10μg), Ceftriaxone (30μg), Piperacillin (100µg), Cefotaxime (30µg), Meropenem (10µg), Ceftazidime (30μg), Levofloxacin (5μg), Tobramycin (10µg), Ofloxacin (5µg) and Ciprofloxacin (5μg).

Bacteriophage sampling

Various crude samples for bacteriophage isolation were collected from different areas in Baghdad city including sewage, farm soil, wastewater, cattle faeces, chicken litter and faeces of sheep during the period from June 2020 to November 2020. The crude samples were mixed in (100 ml) clean test tubes then enfolded by para-film, put in an ice bag and carried to the laboratory on the same day. The samples were put in the refrigerator at 4°C until used.

Phage spot lysis assay

Virulent bacteriophages were selected by phage spotting test on a nutrient-agar. The fashioning of inhibition zones proposed the presence of specific lytic bacteriophages.First, the target bacteria were sub-cultured in Nutrient broth at 37°C for 18-24h. After incubation, a bacterial lawn makes by poured one ml of the bacterial broth onto a nutrient agar plate and spread by the sterile swab. After 20-25 min, the lawn must dry.Then, Ten (10) µl of primary bacteriophage suspension were dropped onto the surface of the bacterial lawn by using a mechanical pipette and were allowed to dry before incubating at 37°C for 18-24h in an inverted state. The next day, if the zone of lysis or plaques was formed at the spot of the primary phage suspension, a lytic and specific phage for the target bacteria was identified[11].

Resultes and Discussion

Identification of the isolates

All isolates appear as Gram-negative and showed negative results for the oxidase test except the isolates that belong to the *Pseudomonas* genus showed positive results to the oxidase test. All *A. baumannii* isolates showed the ability to grow at 44ºC and this inspection was used to differentiate *A. baumannii* from other *Acinetobacter* species which are unable to grow at this temperature degree. Also, weconfirmation the results with Vitek 2 compact system. And this study "agreement with the findings of other recent study carried in Iraq" that recorded this bacteria (*P. aeruginosa, K. pneumoniae and A. baumannii*) as one of the most common microorganisms causing sepsis in burn patients (Almajidy et al., 2020)[12].

Antibiotic susceptibility test

This test showed that 30 out of 42 bacterial isolates collected were highly resistant isolates to most of the conventionally applied antibiotics.The results showed that different bacterial isolates had different antibiotic sensitivity profiles.Figures 1, 2 and 3 shows the percentage of resistant, sensitive and intermediate bacterial isolates to each antibiotic.

The characteristics of the isolated phages

The characteristics of plaque assay of the isolated phages showed that plaques clarity (clear, semi-clear, turbid). In this study, we isolated highly lytic phages that produced obvious inhibition zone on target bacteria and if the plaque not clear or semi-clear we repeated the steps of primary phage isolation on different crude samples. This finding is in line with other studies (Kusradze I et al., 2016)[13]. Table (1) showed that one primary phage can lysis more than one of A. baumannii isolates but not all isolates show inhibition zone, so we isolated four primary phages to lysis all *A. baumannii* isolates.Table (2) showed that one primary phage can lysis more than one of *P. aeruginosa* isolates bunot all isolates show inhibition zone, so we isolated four primary phages to lysis all *P. aeruginosa* isolates.Table (3) showed that one primary phage can lysis more than one of *K. pneumoniae* isolates but not all isolates show inhibition zone, so we isolated three primary phages to lysis all *K. pneumoniae* isolates.Figures (4), (5) and (6) showed phage spot lysis assay of isolated bacteriophages on bacterial lawns of different bacterial isolates.

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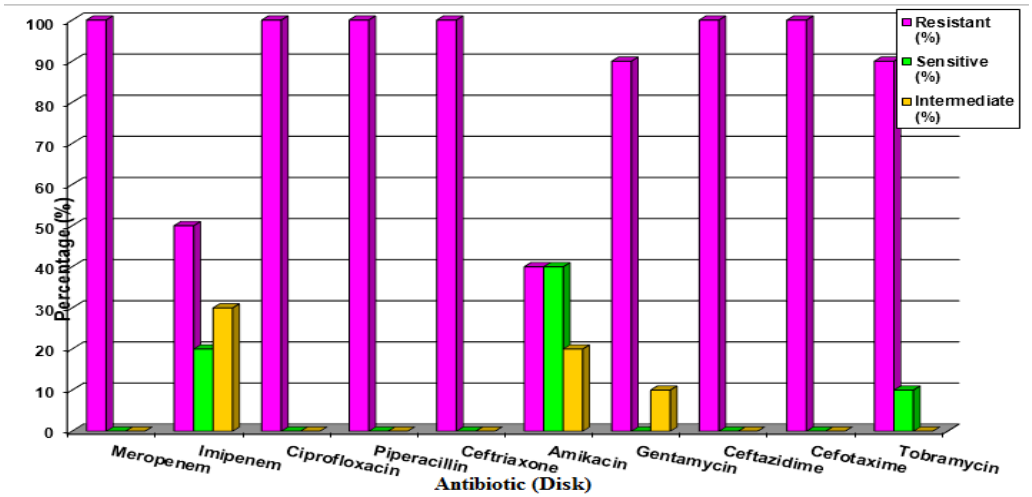

Figure (1): The percentage of resistant, sensitive and intermediate isolates of *A. baumannii* to each antibiotic

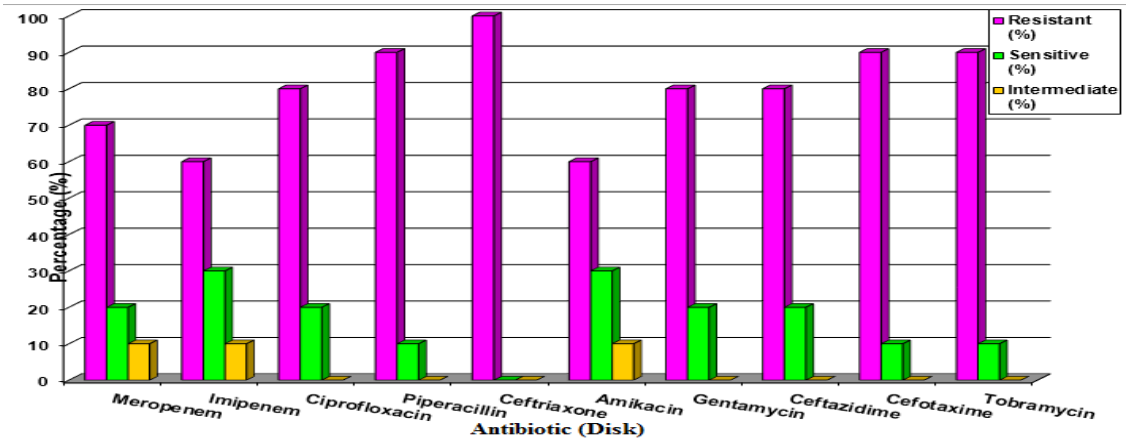

Figure (2): The percentage of resistant, sensitive and intermediate isolates of *K. pneumoniae* to each antibiotic

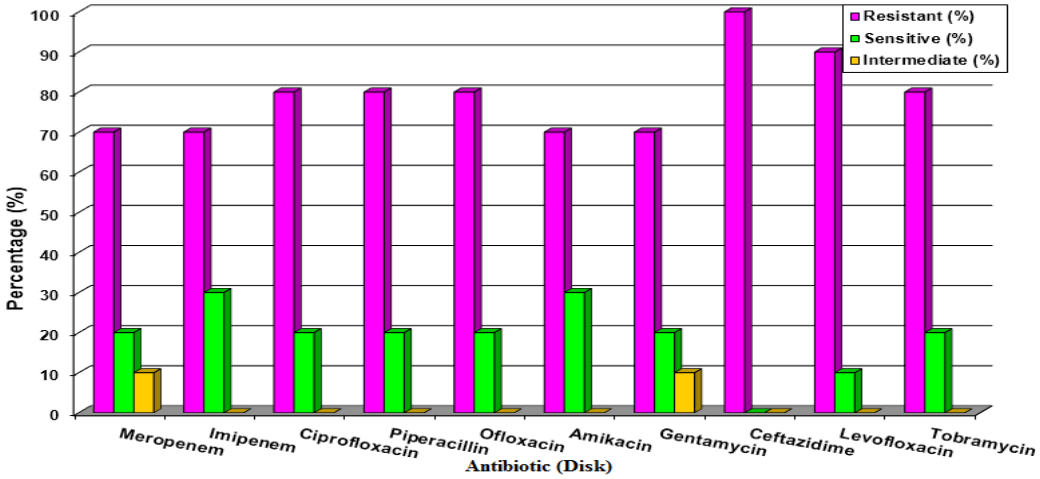

Figure (4): The percentage of resistant, sensitive and intermediate isolates of *P.aeurginosa* to each antibiotic

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Table (1): plaque assay characteristics of the isolated phages to *A. baumannii*

Primary phage(PP)	A. baumannii isolates(A.B)	Plaque clarity
A.BPP1	A.B1	Clear
	A.B2	Clear
	A.B4	Semi-clear
A.BPP2	A.B3	Clear
	A.B5	Semi-clear
	A.B9	Clear
A.BPP3	A.B6	Clear
	A.B8	Clear
A.BPP4	A.B7	Clear
	A.B10	Semi-clear

Table (2): plaque assay characteristics of the isolated phages to *P. aeruginosa*

Primary phage(PP)	*P. aeruginosa*isolates(P.A)	Plaque clarity
P.APP1	P.A1	Clear
	P.A8	Clear
P.APP2	P.A2	Clear
	P.A3	Semi-clear
	P.A7	Clear
P.APP3	P.A4	Semi-clear
	P.A6	Clear
	P.A8	Semi-clear
P.APP4	P.A5	Clear
	P.A10	Clear

Table (3): plaque assay characteristics of the isolated phages to *K. pneumoniae*

Primary phage(PP)	*K. pneumoniae*isolates (K.P)	Plaque clarity
K.PPP1	K.P1	Clear

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	K.P7	Semi-clear
	K.P9	Clear
	K.P10	Semi-clear
K.PPP2	K.P2	Clear
	K.P5	Semi-clear
	K.P6	Clear
	K.P8	Clear
K.PPP3	K.P3	Clear
	K.P4	Semi-clear

Figure (4): phage spot lysis plaques assay of isolated bacteriophages against *A. baumannii* isolates no.3.

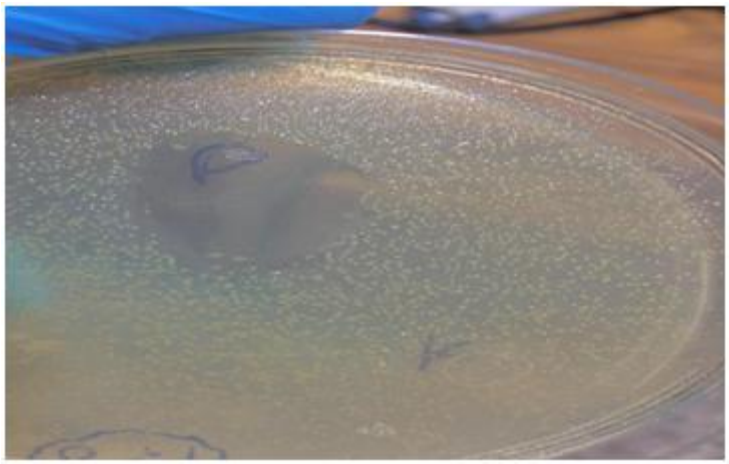

Figure (5): phage spot lysis plaques assay of isolated bacteriophages against *P. aeruginosa* isolates no.2

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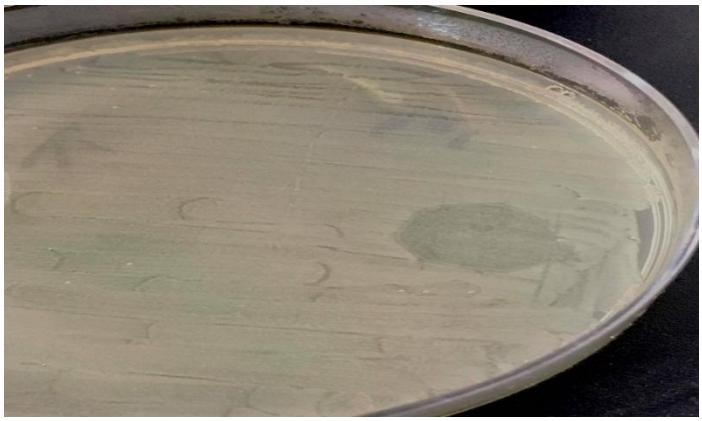

Figure (6): phage spot lysis plaques assay of isolated bacteriophages against *K. pneumoniae* isolates no.5

Conclusions

A. baumannii, *P. aeruginosa* and *K. pneumoniae* bacteria are highly multi-drug resistant bacteria, isolated in Baghdad city from patients suffering from infected skin burns in different places of the body**.**Phages isolation and purification were possible, does not take long, flexible and economic. The isolated phages showed high efficacy in the lysis of MDR bacteria. Formed Phage cocktail was useful to increase the range of effectiveness against MDR bacteria and to tackle the problem of bacterial resistance to bacteriophages

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