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Treatment of *Acinetobacter baumannii* by Specialized ϕTZ Bacteriophage and Studying of pH Effect on Bacteriophage Stability and Efficacy

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Abstract

In general, one of the most important and dangerous pathogens facing human society is the resistance of different types of bacteria to many types of antibiotics. *Acinetobacter baumannii* is one of the most dangerous pathogens because of its prevalence in the hospital environment, being among the normal flora of bacteria inside the body also they are opportunistic bacteria due to their ability to turn into pathogenic bacteria when there is any weakness in the body's immunity thus causes many diseases such as pneumonia, inflammation of the central nervous system and many different diseases. Because of the high ability of *A. baumannii* to acquire genes responsible for resistance to all antibiotics, including carbapenems, from the environment, it has become necessary to discover new methods to limit the spread of this bacteria and treat them. Among these methods is the treatment by bacteriophages then studying the physical properties, and one of the important characteristics that must be studied is the best pH that maintains the stability and effectiveness of the bacteriophage specialized in treating *A. baumannii* (ϕTZ), which belongs to the family Myoviridae. The pH 6.0 was found to maintain the best activity in the lysis of bacteria and stability of Bacteriophage. As well as, it was able to survive 100%.

1. Introduction

Acinetobacter baumannii is an apportunistic pathogen, mainly affecting patients in the hospitals, that has emerged as one of the most troublesome pathogens by its resistant to most antibiotics. *A. baumannii* is the most frequently encountered of the *Acinetobacter* species and DNA groups that oxidize glucose and are not hemolytic. Oxidation of glucose and absence of hemolysis, however, are not sufficient characteristics for identification of *A. baumannii*. The ability to grow at 44℃ will separate *A. baumannii* from the other species [1].

Acinetobacter baumannii is a gram negative, aerobic microorganism and non-fermenting that plays an important role in infecting patient entering the hospital especially in intensive care and burn units [2 & 3]. The ability to grow *Acinetobacter baumannii* at a temperature of 44 degrees Celsius is one of the most important characteristics that distinguish this type from other types of *Acinetobacter*, *A. baumannii* causes a great challenge to researchers because of its resistance to many antibiotics, and it is usually an opportunistic bacteria

that infects most patients in hospitals [1]. *A. baumannii* grows under aerobic conditions and is Gram-negative and most commonly infects patients in intensive care units and burns [2]. As a result of the presence of *Acinetobacter spp*. in the soil and because of its proximity to organisms that produce antibiotics, these bacteria have the great ability to acquire resistance to these antibiotics [4]. The bacteria *A. baumannii* is naturally present in many areas of the human body and therefore because it is opportunistic, it can cause multiple infections in the case of immunodeficiency [5]. "This characteristic has made it into one of the main organisms threatening the current antibiotic era" [2].

Bacteriophage plays an important role in maintaining a balanced ecosystem [6]. Because of the ability of Bacteriophage to eliminate bacteria, therefore it is an effective therapeutic substance [7]. Especially antibiotic-resistant bacteria, which have become a major threat to human health. Treating the many serious diseases that can be caused by the antibiotic-resistant A. baumannii has become an absolute necessity, and therefore the use of Bacteriophage that analyzes these bacteria can be the best solution to treat these diseases [8].

pH is an important factor in the storage of therapeutic phages, so it was necessary to study the effect of pH on the phages that have the ability to analyze these bacteria.

2. Experimental Procedure

2.1. The Strains Collections

The samples were collected from Egypt hospitals Between February and october 2016, The samples were collected from patients admitted to different wards with different infections. The specimens were collected and transported according to [9] under aseptic conditions quickly to the Microbiology Laboratory at Faculty of Science, Zagazig University where the study was carried out.

2.2. Identification by Analytical Profile Index (API) 20E Strips

The Analytical Profile Index (API) 20E strips obtained from Biomerieux, France were used as biochemical system for identification of *A. baumannii*. The API 20E strip consists of 20 micro-tubes containing dehydrated substrates.

2.3. Antibiotic Sensitivity Test for Bacterial Isolates

In this investigation, seventeen commercially prepared antibiotic discs (6 mm in diameter) belonging to twelve different groups were chosen for investigating their potency against bacteria isolated from clinical specimens. These antibiotic classes are the most commonly used in human medicine, veterinary medicine, animal husbandry and in agriculture in Egypt. The discs were obtained from Oxoid, UK. In this test, using the standard Kirby-Bauer disk diffusion method [10].

2.4. Phage Isolation, Purification and Propagation

Different Egyptians hospital waste water samples were used for isolation of active phages by culture-enrichment method described in [11], with simple modifications.

2.5. Spot Test and Plaque Assay

The spot test method as well as the plaque assay [12] was used to screen for the presence of lytic phage activity in the resultant concentrates using clinical *A. baumannii* strains.

2.6. Electron Microscopy

Morphologic assessments of the isolated phages were examined by transmission electron microscopy using Na-phosphotungstate or 2% aqueous uranyl acetate (pH 4.8) for stained.

2.7. Effect of pH Value on the Survival of Isolated Phages

The effect of pH degree on the survival and stability of phages was determined using LB liquid medium of various pH degrees. The filtered phage suspension was diluted approximately to 10^6 PFU/ml. Phages were diluted in test tubes containing 9 ml; of liquid medium adjusted to various pH degrees using 0.1 N HCl and/or 0.1 N NaOH (i.e. 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13) according to [13]. After incubation of the mixtures at 4℃ overnight, the residual phage activity was determined by plaque assay technique [13].

3. Results and Discussion

Tables (1) total of 910 isolates Gram negative bacilli isolates were collected from the 1350 clinical samples. Depend on the morphological characterization and biochemical, 18 (2%) isolates were identified as suspected *Acinetobacter spp*., which detected as non-lactose fermenter and oxidase negative coccobacilli (appeared as pale colonies on MacConkey agar). (Figure 1 and Table 1), and only 13 (1.4%) isolates were identified as suspected *A. baumannii* at very good identification level by API 20E system (Table 2).

Table (1). Distribution of bacterial isolates according to source of isolation.

	Respiratory Tract Infections		Urinary Tract Infections		Wound Infections		Blood Strea Infections		Total	
Type of bacteria	No.	%	No.	%	No.	%	No.	%	No.	%
Non *Acinetobacter* isolates	290	31.8	217	23.8	197	21.6	188	20.6	892	98
***Acinetobacter* isolates**	8	0.87	5	0.54	3	0.32	2	0.21	18	2
Total bacteria isolates	298	32.67	222	24.34	200	21.92	190	20.81	910	100

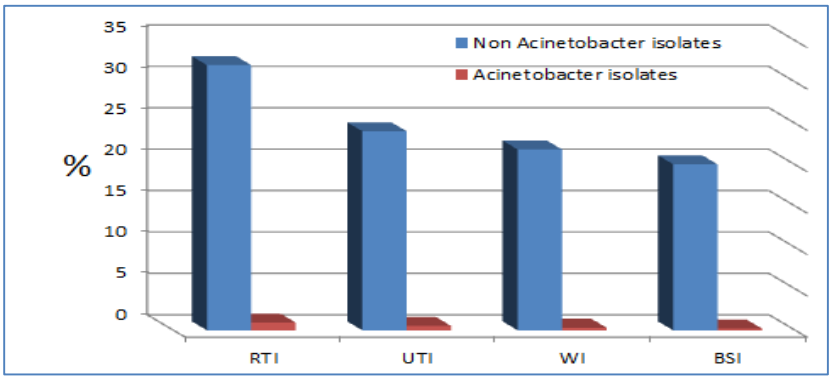

Figure (1). Distribution of bacterial isolates according to source of isolation, RTI: Respiratory Tract Infection, UTI: urinary Tract Infection, WI: Wound Infection, BSI: Blood Stream Infection.

Moreover, Table (2) revealed thatamong 910 Gram-negative bacteria only 13 (1.4%) isolates were identified as suspected *A. baumannii* at very good identification level by API 20E system.

Table (2). Incidence of bacterial isolates recovery and identification from clinical samples collected from Egyptian hospital.

Total No. of isolates	**No .of (+ve) growth on Macconkey**	**No. of *Acinetobacter SPP.***	**%**	**No. of *Acinetobacter baumannii***	**%**
1219	910	18	2	13	1.4

3.1. Resistant Bacterial Isolated

All *A. baumannii* isolates was showed resistant against all antibiotics used with different percentages except Ampicillin/sulbactum, Amikacin, imipenem, polymyxin B and Norofloxacin (Figure 2), and these caused increasing problems with resistance against antibiotics in common pathogenic bacteria and the concern about spreading of antibiotic resistance in the environment [14]. In this research the data show 94.1% resist against the antibiotics and these results agree with [15-16].

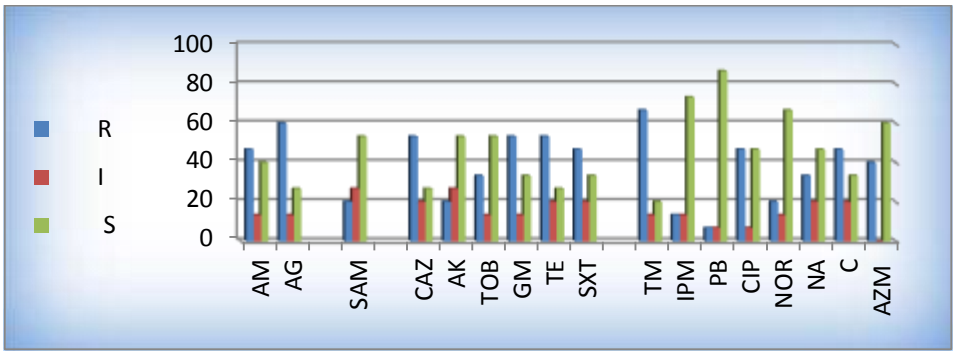

Figure (2). Comparative susceptibility of *Acinetobacter* baumannii isolates against different antibiotics.

3.2. Isolation of Lytic Bacteriophages

Phages of *A. baumannii* were isolated from wastewater of Egyptian hospitals using spot test technique and double layer technique. Several different plaques were isolated using 13 strains of *A. baumannii*. The strain Ab531 isolated from wound infection showed sensitivity to phages and was selected as a phage multiplication strain. Phages can be found anywhere bacteria grow [17]. After infecting the bacteria with the phage and multiplying inside it, the enzymes secreted by the phages analyze the bacterial cell wall, where these enzymes bind to specific sites in the peptidoglycan and break certain bonds, thus breaking the peptidoglycan, and then decomposing the bacterial cell wall, where the halos surrounding the center of the plaques indicate the presence of phage that capable of degrading exopolysaccharide (EPS) formed by *A. baumannii* [18].

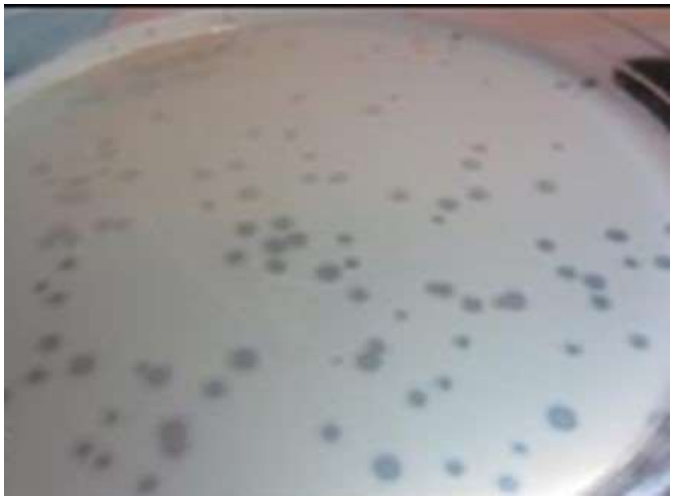

Figure (3). Plaque morphology of the A. *baumannii* bacteriophage.

Table (3). Isolation of *Acinetobacter baumannii* bacteriophages from different sewage source.

No. of source	Phage source	N0. of samples	Formation of lytic area
A	Sewage treatment plant in Alqnayat (AL-Zagazig)	4	-
B	Sewage treatment plant in Tenth of Ramadan	3	-
C	Sewage treatment plant from Abu Khalil (AL-Zgazig)	6	-
D	Sewage treatment plant from Cairo.	1	-
E	Sewage treatment plant from the city hospital (AL-Zagazig)	4	++
F	Sewage treatment plant from the univercity hospital station (AL-Zagazig)	4	++++

Table (4). Formation of lytic area, titer and plaque morphology of the inrechment of sewage source with different isolatesof *Acinetobacter baumannii*.

No. of Bacteria	Lytic area	source	Titer	Morphology	
				Diameter	Apperence
Ab9	+	F	$1.3X10^{6}$	4mm	Turbid center& turbid area
Ab17	+	F	$2.2X10^{8}$	1mm	Clear center&clear area
Ab37	+	F	$6.0X10^{5}$	5mm	Turbid center& clear area
Ab59	-	F	-	-	-
Ab77	+	F	$1.3X10^{8}$	0.5mm	Clear center& clear area
Ab184	+	F	$2.1X10^{6}$	-	-
Ab206	+	F	$2.6X10^{8}$	1mm	Clear center& clear area
Ab531	+	F	$1.8X10^{10}$	6mm	Turbid center & clear area
Ab613	+	F	$1.5X10^{8}$	6mm	Turbid center& clear area
Ab859	+	F	$1.2X10^{8}$	4mm	Turbid center & turbid area
Ab1124	+	F	$4.0X10^{5}$	0.5mm	Clear center & clear area
Ab1173	-	F	-	-	-
Ab1225	+	F	$1.1X10^{6}$	1mm	Clear center & clear area

3.3. Electron Microscopy

Electron microscopy of the isolated *Acinetobacter baumannii* phages particles revealed that the ØTZ *A. Baumannii* bacteriopage was classify into morphology have an icosahedral head (63 × 53 nm) in diameter with a contractile tail (105nm) in length belonging to the Myoviridae (Figure 4), The phage was morphologically similar to earlierisolated phage AP22 [3], according to International Committee on Taxonomy of viruses (ICTV) [19].

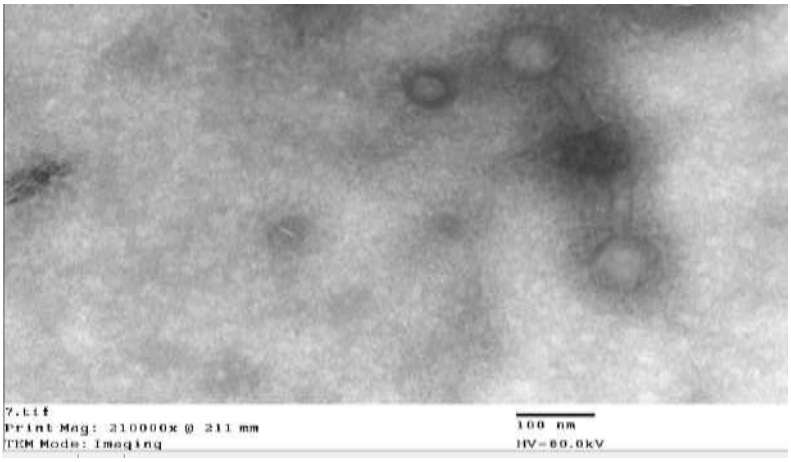

Figure (4). Electron micrographs of isolated *A. baumannii ØTZ* phages.

3.4. Effect of pH Value

The ability to adsorption the phages on outer surface of bacteria is directly affected by the pH of the medium in which it is located, and therefore the pH indirectly affects the survival of phages [20]. In this study *Acinetobacter baumannii bacteriophages* ØTZ remaind steble within 24h between pH 4 and 11. The maximum stability of the phage was observed at a pH between 6 and 7. It is noted from Figure (5) and Table (5) that the decrease in the pH levels led to the decrease in the bacteriophage multiplication level. The reason for this is due to the acid denaturation of the protein, which in turn affects the persistence of specialized phages for *A. baumannii* infection. pH stability is an important factor in the storage and survival of therapeutic phages.

Table (5). Effect of pH values on different *Acinetobacter baumannii* bacteriophages isolates.

pH Degree	ØAbZ1		
	Pfu/ml	Log. Pfu/ml	Surv. %
2	0.0	0.0	0.0
3	0.0	0.0	0.0
4	2.1×10^4	4.32	62.6
5	3.8×10^4	4.57	66.3
6	7.9×10^6	6.89	100
7	2.7×10^6	6.43	93.3
8	4.0×10^4	4.60	66.7
9	2.1×10^4	4.32	62.6
10	1.1×10^4	4.04	58.6
11	3.0×10^3	3.47	50.3
12	0.0	0.0	0.0
13	0.0	0.0	0.0

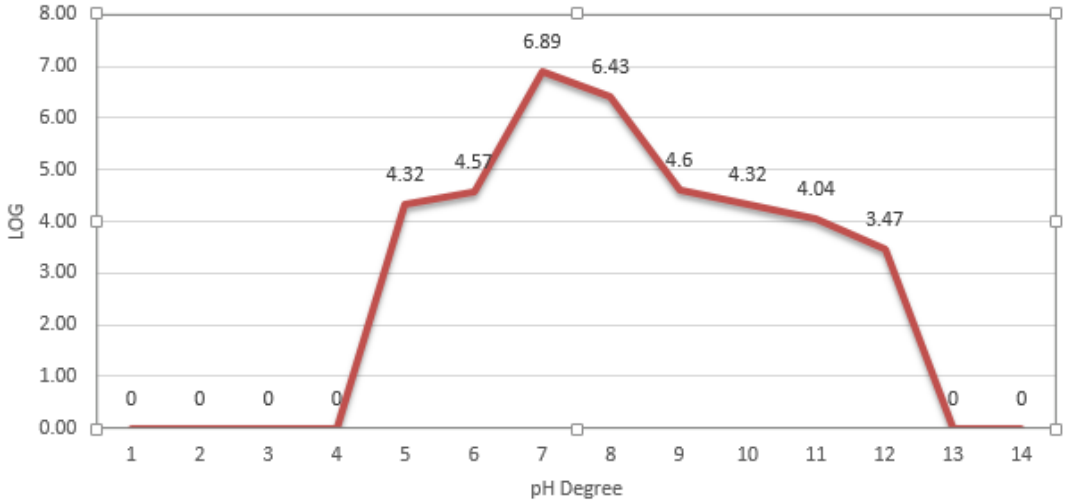

Figure (5). Effect of PH values on different *Acinetobacter baumannii* bacteriophage isolates.

4. Conclusions

In this research, *A. baumannii* bacteria resistant to seventeen commonly used medically antibiotics were isolated and identified. On the other hand, the ØTZ bacteriophage, which belongs to the Myoviridae family, specialized for the treatment of these bacteria, was isolated, and the best pH degree was studied, through which it was possible to maintain the activity and effectiveness of this bacteriophage against *A. baumannii* for the longest possible period of time for the purpose of using it as a treatment (the maximum stability of the phage was observed at a pH in between 6 and 7).

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