RESEARCH ARTICLE

ISSN: 0331 - 670X

https://doi.org/10.51412/psnnjp.2023.5

The Nigerian Journal of PHARMACY
AS MEN OF HONOUR WE JOIN HANDS
THE OFFICIAL ORGAN OF THE PHARMACEUTICAL SOCIETY OF NIGERIA FOUNDED 1927

Isolation and Characterization of Novel Cocktail Phages of Multidrug-Resistant (MDR) *Acinetobacter Baumannii*

Nkechi V. Enwuru[1], Jachike T. Nzekwe[1], Bisola O. Ojo[1*], Abdul-Rahman Usman[2], and Folake R. Peters[2].

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, University of Lagos.
[2]Department of Medical Microbiology & Parasitology, Lagos University Teaching Hospital, Idi-Araba, Surulere, Lagos, Nigeria.

ARTICLE INFO

Article history:

Received 22 November 2022
Revised 25 January 2023
Accepted 1 March 2023
Online Published 31 March 2023

Keywords:

Mdr *A. Baumannii*,

Lytic *A. Baumannii* Phage,

Optimized Ph,

And Temperature Stable Phages.

* *Corresponding Author:*
boladapo@unilag.edu.ng
+2349091178874
https://orcid.org/0000-0003-0330-8599

ABSTRACT

Background: *Acinetobacter baumannii* is a nosocomial organism, classified as a critical pathogen with multidrug-resistant (MDR) challenges thus, it is urgently necessary to develop novel antimicrobial strategies. Phage therapy is an alternative and promising strategy, a highly effective biocontrol agent against MDR *A. baumannii*. This study characterizes three phages of MDR *A. baumannii*.

Method: *Acinetobacter baumannii* was isolated from clinical wound specimens and was identified using VITEK® 2 system (Biomerieux, France). The antibiotic susceptibility profile was determined according to the Clinical and Laboratory Standard Institute (CLSI). Phages specific to MDR *A. baumannii* were isolated from sewage and irrigation channel samples, then tested for pH and temperature stability. **Results**: Among the *A. baumannii* strains isolated, five strains (Ab 140, Ab 150, Ab 333, Ab 1289, and Ab 976) showed remarkable resistance to antibiotics tested, with the resistance rate ranging from 90 to 40 %, respectively. Three lytic phages specific to Ab 140, Ab 150, and Ab 333 were isolated among five MDR *A. bamannnii* isolates used for phage isolation. Lytic Phages specific to Ab 140 and Ab 150 produced clear big plaques with halo-like appearances around the inhibition zone, while Ab 333 produced clear and small size plaques. The phages were designated TJ 140, TJ 150, and TJ 333 and were stable in various pH and temperatures with a high survival rate in pH of 5.0 to 7.0 and 20 to 60 °C, respectively.

Conclusion: The isolated phages exhibited strong lytic activity against MDR *A. baumannii* isolates tested and are stable in various pH and temperature ranges. They had no lytic effect on the heterogeneous strains and are good potential candidates for therapeutic applications.

1. Introduction

Antimicrobial resistance has become a major global concern. The emergence of multidrug-resistant (MDR) organisms and the slowing down of the pipeline to generate new antibiotics is a critical concern in treating bacterial infections[1]. Antimicrobial resistance leads to increased morbidity and mortality, increased healthcare costs, and lower quality of life for patients[3].

The World Health Organization (WHO) rates antimicrobial resistance as one of the top ten global health threats in 2019[4]. Carbapenem-resistant *Acinetobacter baumannii* (CRAB) was placed on the urgent and critical list of organisms by the US CDC (United States Centre for Disease Control and Prevention) and WHO, respectively, as a pathogen that urgently requires research and development of new antimicrobial strategies[5,6]. Furthermore, the current WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report states that resistance of *A. baumanii* to Carbapenems in bloodstream infections is high, depicting a very dire

scenarioho[7].

Acinetobacter baumannii is a nosocomial opportunistic pathogen commonly implicated in infections of critically ill and immunocompromised patients in healthcare facilities and hospital settings. The infections can range from skin and soft tissue infections, bloodstream infections, hospital-acquired and ventilator-associated pneumonia, secondary meningitis, and urinary tract infections, all linked with high morbidity and mortality rates[8]. This microorganism has a high propensity to develop resistance to antimicrobials via its various virulence factors (porins, capsules, cell wall lipopolysaccharides, enzymes, iron acquisition systems, and biofilm formation) [9] resulting in the development of severe and persistent infections. These factors also significantly impact its environmental persistence, host-pathogen interplay, and immune evasion [10].

Acinetobacter baumannii is a member of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, A. baumannii, Pseudomonas aeruginosa*, and *Enterobacter species*) group of pathogens which are prominent for causing frequent and hard-to-treat healthcare-associated infections[11].

Multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) strains have been observed globally [12,13]. Historically, carbapenems are the most potent and reliable β-lactam antibiotics fortreatingf serious infections caused by *A. baumannii*[14]. However, the prevalence of carbapenem-resistant *A. baumannii* (CRAB) seriously compromises the use of carbapenems in the fight against such infections worldwide (Jeon *et al.* 2016). Carbapenem-resistant *A. baumanii* is usually resistant to almost all available antimicrobials. Colistin, another antimicrobial agent, has serious toxicity though it is effective against most CRAB and is a last-resort treatment for this reason. Consequently, low treatment options and considerable mortality (about 50%) are associated with CRAB[15]. A study has shown that the average resistance rate of *A. baumannii* to carbapenems is over 70%. The last resort treatment of carbapenem-resistant *A. baumannii* (CRAB) involves combinations of colistin and tigecycline, but the safety and efficacy are still unclear [16]. Hence there is a need for urgent alternatives against CRAB infections.

Phage therapy is a potential alternative. Bacteriophages (phages) are viruses that infect bacteria and are numerous in the biosphere [17]. Phages have been recorded as therapeutic agents to treat bacterial infectious diseases in humans and animals. The use of Phage therapy is a promising tool for controlling drug-resistant bacteria [18]. Several *A. baumannii* bacteriophages have been isolated and characterized globally[19,20]. It has been used experimentally either alone or in combination with antibiotic agents (Tigecycline/Colistin). A study that combined MDR A. baumanii phage with Tigecycline reported synergistic effects and better antibacterial activity than phage or antibiotic alone [21].

Compassionate phage therapy for patients with debilitating diseases complicated by CRAB infections, including patients with critical COVID-19 infections with secondary pulmonary CRAB infections, showed reduced bacterial burdens [22]. A single phage combined with either Tigecycline or Polymyxin E saved the life of 88yr old Chinese man with hospital-acquired pneumonia caused by CRAB [23].

Phage preparations developed for Western bacteria isolates may not apply to the tropical setting, since microorganisms differ in different locations. This study is justified by the urgent need to develop tropical-specific phages to be tried on Nigerian MDR *A baumannii* isolates to diversify the treatment of diseases caused by MDR *A. baumannii*.

2. Method

2.1 Materials

Tryptone Soya agar (HiMedia®, India), Mueller Hilton agar (HiMedia®, India), SM Buffer (HiMedia®, India), $CaCl_2$ (Merck®, Germany), SM Buffer (HiMedia®, India), 0.22 μm Membrane filters (Merck®, Germany), Gram-negative antimicrobial susceptibility discs (Oxoid®, UK).

2.2 Bacterial isolates, Identification, and Antibiotic Susceptibility test

Bacteria were isolated from clinical wound samples deposited at Medical Microbiology Laboratory, Lagos University Teaching Hospital, and identified using VITEK 2 system using the Gram-negative identification (GN ID) card according to the manufacturer's instruction (BioMerieux, France)[24]. The antibiotic susceptibility test was carried out using the Kirby-Bauer method. In this assay, ten types of antibiotic disks were used, and these are amikacin (30 μg), tetracycline (30 μg), ceftazidime (30 μg), imipenem (10 μg), meropenem (10 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), ampicillin/sulbactam (20/10 μg), ciprofloxacin (5 μg) and colistin (10 μg). The 0.5 McFarland standards of bacteria isolates were prepared, inoculated, and spread by a sterile swab on Muller-Hinton agar Medium. Antibiotic discs were then placed on inoculated agar plates by sterile forceps. The plates were left in an incubator upside down at 37 °C for 18-24 hr [25].

2.3 Phage enrichment and isolation

Sewage water samples from the University of Lagos' sewage treatment center and water samples from the drainage water channel beside Lagos University Teaching Hospital (LUTH) were pooled and centrifuged at 6000 g for 10 min at 4 °C. The supernatant was filtered using a 0.22 µm membrane filter. Forty milliliters (40mL) of filter-sterilized water samples were mixed with an equal volume of sterile double-strength tryptone soya broth (TSB) with 100 µL of 24 hr bacterial culture of five MDR *A. baumannii* (Ab 140, Ab 150, Ab 333, Ab 1289, and Ab 976). The mixture was incubated overnight at 37 °C with continuous shaking at 200 r.p.m. After the culture was centrifuged at 6000 g for 20 min at 4 °C, the supernatant was filtered through a 0.22 µm membrane filter.

2.4 Phage Spot titration

The presence of lytic phages was tested by the spot method and identified by plaque assay. For the spot test, modified TSA plates were divided into ten sections, 100 µL of 4 hr culture of the test indicator *A. baumannii* strains (Ab 140, Ab 150, Ab 333, Ab 1289, and Ab 976) were mixed with 4ml modified TSA at 45 °C. The content of the mixture was poured as an overlay on the hard agar aseptically, the plates were left slightly opened to set under the biosafety cabinet. A tenfold serial dilution of the cocktail phages of *A. baumannii* was carried out using 900 µL of phage buffer solution (10 mM of Sodium chloride, 8 mM of Magnesium sulfate, and 0.01 % gelatin). A 10 µL of the stock and the diluted phage solutions (tubes 1-9) were spotted on the surface of the sections of the agar plates. The plates were left slightly open under the biosafety cabinet to dry. After, the plates were incubated at 37 °C for 24 hrs. After incubation, a clear zone indicated the presence of a lytic phage against the indicator strains [26].

2.5 Optimization and Characterization of Isolated phages

The phage isolates were characterized using plaque assay. Thus, plaque characteristics were determined using top layer plaque assay and according to the following parameters: a) Diameter (mm) of the plaque. b) Shape of the plaque. c) Depth of the plaque. d) The plaque margin. e) Clarity or turbidity of the plaque. The clearest and largest plaques were selected. While the small, or turbid plaques were subjected to optimization by conducting serial passage on top-layer plaque assays. At each run, the best of the plaques, in terms of the above-mentioned parameters, were selected to acquire better virulence characteristics of the isolated lytic phages [27].

2.6 Phage purification and amplification

An optimized clear plaque was picked from the agar overlay with a sterile tip and resuspended in 500 µL sterile SM buffer (0.1M NaCl, 8 mM MgSO4, 7H2O, 50 mM Tris-HCl pH 7.5). The mixture was incubated at 4 °C overnight. Subsequently, 100 µL of the mixture was used for tenfold serial dilutions in 900 µL of SM buffer. A 100 µL of log phase bacterial strains of *A. baumannii* (Ab 140, Ab 150, and Ab 333) was used to prepare bacteria lawns, and 10 µL of phage dilution were spotted on the surface of the bacterial lawns, and plates were incubated overnight at 37 °C. The single plaque isolation was repeated for four rounds to obtain purified plaques [17].

For phage amplification**,** 10 mL sterilized tryptone broth was inoculated with 100 µL of overnight indicator organisms. Then 50 µl of purified specific phage was added. A 10 mL TSB was inoculated with 100 µL of test bacteria and incubated alongside as a control. When the lysis was observed, the culture was centrifuged at 3500 rpm for 20 mins. After, it was filter sterilized using a 0.2 µm pore-size membrane filter.

2.7 Determination of Phage Titre

The phage titer was determined by a tenfold serial dilution of 100 µL of purified phage solution in 900 µL of phage buffer solution. Appropriate dilutions were spotted on a double-agar layer plate marked into 12 sections with the appropriate bacteria host. After incubation for 12 to 16 hrs at 37 °C, the plate which contains fewer plaques derived from single phages was counted and the plaque-forming unit per ml was calculated. [26]

2.1 Survival and Stability of TJN-1 Phages

Survival of the phages within some periods was evaluated including their stability in a range of temperature (4-70 °C) and pH (1.5-10) was performed. Briefly, 500 µL of the purified phages in suspension (TJN-1 140, 150, and 333) ($1x10^9$ pfu/ml) of SM buffer (100 mM NaCl, 25 mM Tris HCl, 8 mM MgSO4, 0.01 % w/v gelatin) were filled in sterile brown bottles and stored for 12 months at 4 °C. The phages were evaluated for survival for 3 months, 6 months, 9 months, and 12 months intervals, respectively by counting the titer of surviving phages using the double-layer agar plate method. A 100 µL of each of the phages ($1x10^8$pfu/ml) was inoculated in a series of tubes containing 900 µL SM buffer and incubated at 20 °C, 37 °C, 40 °C, 50 °C, 60 °C, and 70 °C for 1 hr respectively, and then surviving

phages were quantified by soft agar assay. To determine the pH, the pH of SM buffer was adjusted to the required pH using Hana standard buffer (1.5, 3, 5, 7, 9, and 10), 100 µL of phage suspensions (TJN-1 140, 150, and 333) ($1x10^8$ pfu/mL) were inoculated in a series of tubes containing the adjusted SM buffer solution and incubated at 37 °C for 1 hr respectively. After incubation, phage suspension was immediately serially diluted in SM buffer (pH 7.5), and the phage titer was determined by the double-layer agar plate method for every treated sample. The phage survival rates were expressed as percentages of viable phage in suspensions[26,28].

3. Results

3.1 Antibiotics sensitivity of host bacteria strains

Among the ten *A. baumannii* isolated from wound specimens, five were multidrug-resistant. The *A. baumannii* (Ab 1298) was 90 % resistant amongst the ten antibiotics tested and was susceptible to only colistin. The strain, Ab 976 was 80 % resistant and was susceptible to colistin and imipenem. The three strains of *A. baumannii* (Ab 333, Ab 150, and Ab 140) were 40%, 50%, and 40% resistant respectively. They were resistant to Ceftazidime, Cefuroxime, Cefixime, Augmentin, and Nitrofurantoin but were susceptible to Gentamycin, Ofloxacin, Ciprofloxacin, Imipenem, and Colistin. The resistance rate of antibiotics of *A. baumannii* studied ranged from 90 % to 40 % respectively as shown in Figure 1 below.

Table 1: Antibiotic Susceptibility Test

Disk Code	Drug Name	Drug Load	Ab150	Ab140	Ab333	Ab976	Ab1289
CAZ	Ceftazidime	30UG	R	R	R	R	R
CRX	Cefuroxime	30UG	R	R	R	R	R
GEN	Gentamicin	10 UG	S	S	R	R	R
CXM	Cefixime	5 UG	R	R	R	R	R
OFL	Ofloxacin	5 UG	S	S	R	R	R
AUG	Augmentin	30 UG	R	R	R	R	R
NIT	Nitrofurantoin	300 UG	R	S	R	R	R
CRP	Ciprofloxacin	5 UG	S	S	R	R	R
IMP	Imipenem	10 UG	S	S	R	R	R
CT	Colistin	10 UG	S	S	S	S	S

Figure 1: Antibiotic Susceptibility test showing percentage susceptibility across the five multidrug-resistant strains.
*R- Resistant, *S- Susceptible CPR= Ciprofloxacin, GEN= Gentamicin, CAZ= Ceftazidime, CFX= Cefuroxime, IMP= Imipenem, OFL= Ofloxacin, AUG= Augmentin, NIT= Nitrofurantoin, CT= Colistin.

3.1 Phage Isolation

Five MDR *A. baumannii* isolates (Ab 1298, Ab 976, Ab 333, Ab 140, and Ab 150) were used for phage isolation. Phages specific to MDR *A baumannii* strain (Ab 333, Ab 140, and Ab 150) were isolated from sewage and drainage water channels. The phages were designated TJN 140, TJN 150, and TJN 333. The phages exhibited potent lytic activity against MDR *A baumannii,* Figure 2 shows Ab 150, Ab 140, and Ab 333 isolates with clear large to medium plaques that measure1-2.5 mm in diameter with a halo zone around the plaques except for the TJN 333 phage.

Table 2 Phage Spot Assay

Specific vulnerable bacteria biotype (*Acinetobacter baumannii*) with sample code	Spot of Phage plaques from tenfold serial diluted phage solution								
	Stock	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
TJ 333	+	+	+	+	+	+	+	+	+
TJ 150	+	+	+	+	+	+	+	+	-
TJ 140	+	+	+	+	+	+	+	+	+

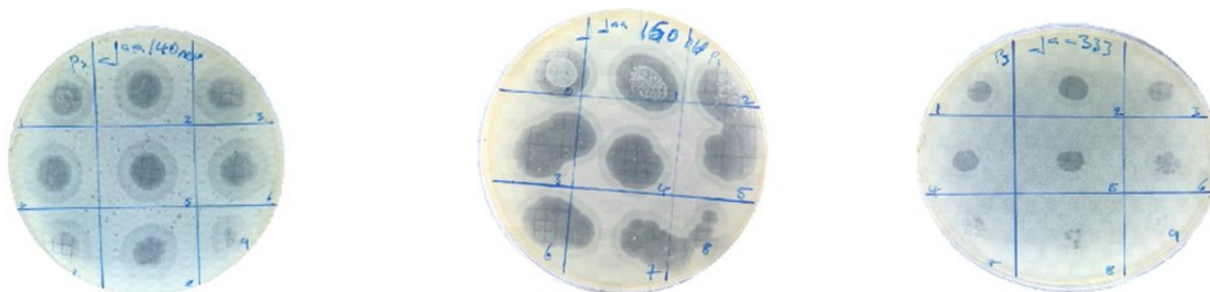

Figure 2: Spot test of isolated *A. baumannii* phages designated as TJN-1 140, TJN-1 150, and TJN-1 333 respectively.

3.1 Physio-morphological characteristics of isolated and optimized TJN-1 phages

The characteristics of the TJN-1 Phages' plaque size range from 0.4 to 3.0 mm, the plaque clarity ranges from semi-turbid or semi-clear to clear, and the phages-maintained a round shape and regular margin. TJN-1 140 and 150 produce a halo zone around each plaque, however, TJN-1 333 does not produce a halo zone. Morphologically, eight (8) phages specific to 3 *A. baumannii* bacteria were isolated. Most of the isolated phages were highly lytic and produced an obvious inhibition zone on target *A. baumannii* bacteria as seen in Figure 3. The titer of the lytic phages isolated and optimized to *A. baumannii* was amplified and measured by using a top-layer plaque assay. Most phages reached high titers ranging between 10^6 -10^8 pfu/mL using top layer plaque assay. The optimized specific and lytic phages were shown to be able to completely lyse the bacterial host in whatever manner of application of phages as demonstrated in Figure 3.

Table 3 Morphological characteristics of TJN-1 Phages

Phage symbol	Plaque size (mm)		Plaque clarity		Plaque shape		Margin cut		Halo zone
	Before	After	Before	After	Before	After	Before	After	
TJN333 P1	0.4	1.0	Semi-clear	Clear	Round	Round	Un-obvious	Regular	-
TJN333P2	1.0	1.5	Clear	Clear	Round	Round	Regular	Regular	-
TJN140 P1	1.5	2.0	Clear	Clear	Round	Round	Regular	Regular	+
TJN140 P2	2.0	3.0	Semi-turbid	Clear	Round	Round	Regular	Regular	+
TJN140 P3	2.5	4.0	Semi-turbid	Clear	Round	Round	Regular	Regular	+
TJN150 P1	2.0	3.0	Clear	Clear	Round	Round	Regular	Regular	+
TJN150 P2	3.0	4.0	Semi-turbid	Clear	Round	Round	Regular	Regular	+
TJN 150P3	2.0	3.0	Semi-turbid	Semi-Clear	Round	Round	Regular	Regular	+

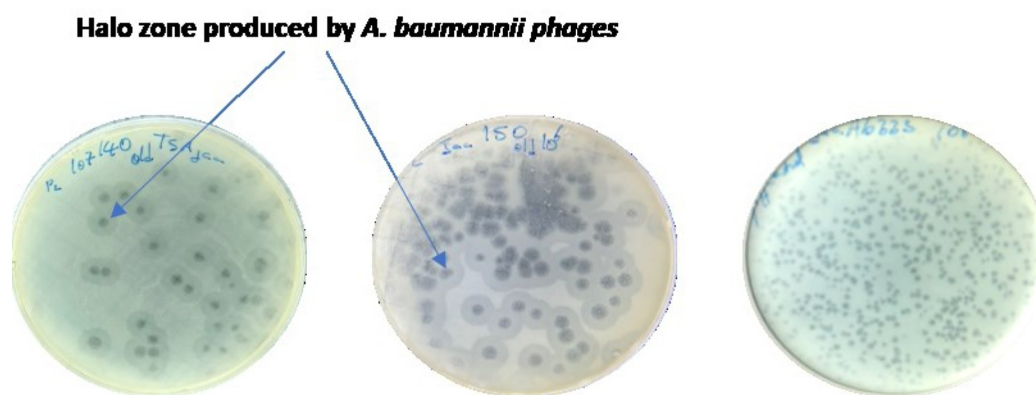

Figure 3: Double-layer plaque assay to show the morphology of TJN-1 Phages 140, 150, and 333.

3.1 Survival of Phages during storage and Stability under different pH, Time, and Temperature

The pH at which the three phages were most stable is pH 6 as shown in Figure 4. The phages' survival rate in pH 1-4 is very low; however, the phages fairly survive at pH 5 and pH 7 except for TJ 333 and TJ 140 respectively.

From Figure 5, all the phages are stable and survive well at 40 °C. At 20 °C, 30 °C, and 50 °C, TJ 333 and 150 phages survived better than TJ 140, with the TJ 140 survival rate of 60 %, 80 %, and 20 %, respectively. However, only the TJ 140 phage was stable and survived well at 60 °C. All the phages were eliminated at 70 °C and 80 °C respectively.

Among the three phages evaluated for survival at pH 7 at 4 °C for a period of one year, TJ 140 survived better, followed by TJ 150, while TJ 333 is the least survived phage.

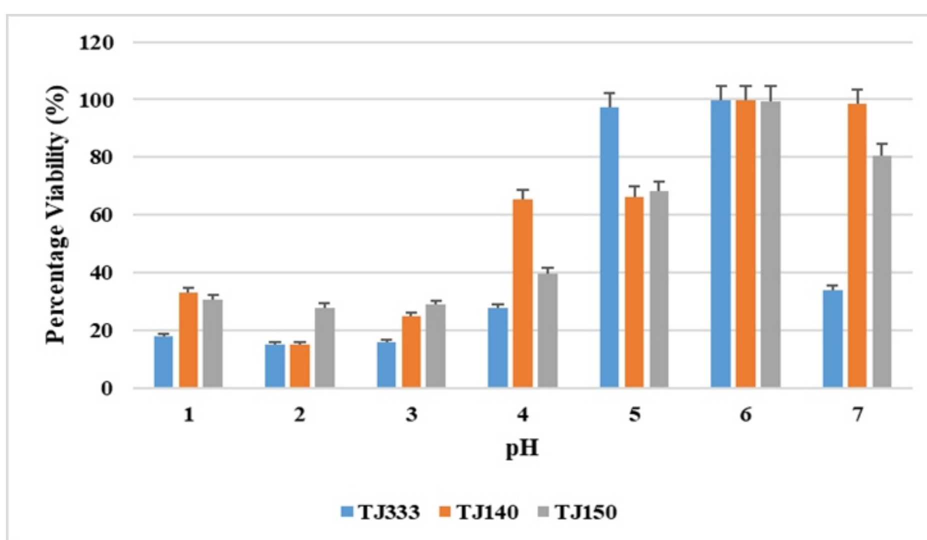

Figure 4: The rate of survival of Phage TJ 333 in different pH values

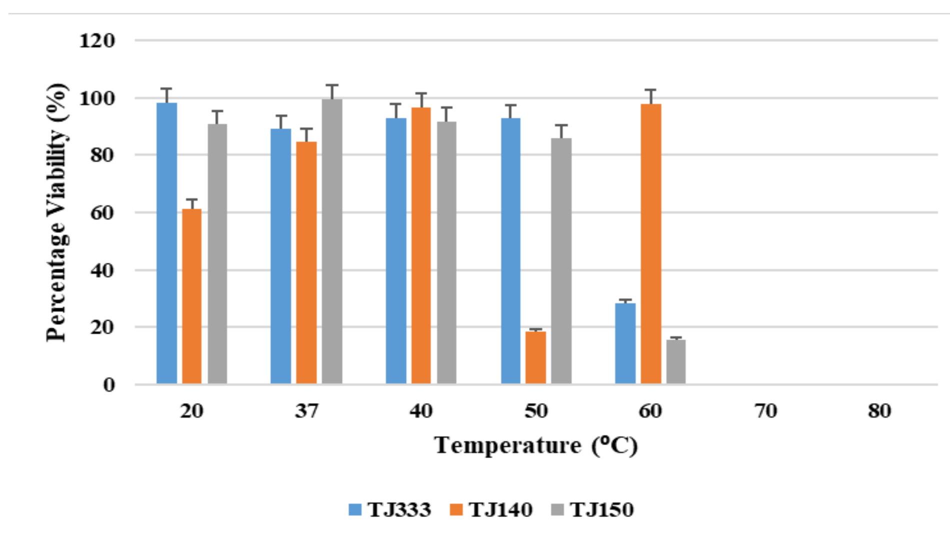

Figure 5: **The rate of survival of Phages (TJ 333, TJ 140, TJ 150) at different temperatures**

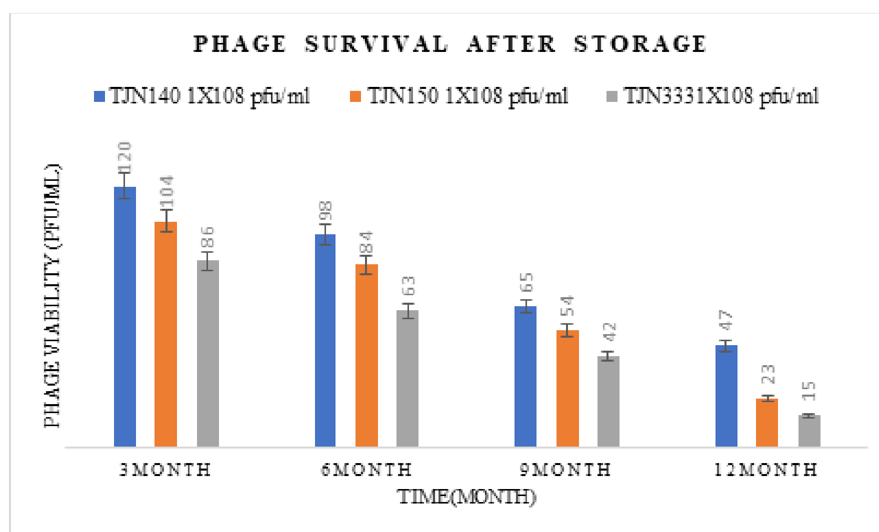

Figure 6: Survival of phages after one year of storage

4. Discussion

Acinetobacter baumannii is a well-known multiple drug-resistant organisms responsible for various debilitating infections globally [29]. In the Coronavirus disease (COVID-19) era, *A. baumannii* could result in secondary bacterial infections, leading to an increased mortality rate in at-risk patients [30]. In Nigeria, although there is no effective reporting on the effects of this organism [12]; its clinical significance cannot be overlooked as several studies reported the resistant mechanisms of this organism from the different parts of the region with very high resistant rates observed [12,31,32]

In this study, two MDR (Ab 140 and Ab150) and three XDR and CRAB (Ab 976, Ab 1289, and Ab 333) *A. baumannii* identified from clinical wound samples were selected for bacteriophage isolation and characterization. Their drug resistance rate was less than 100% but ranges from 90 % to 40 %, and this agrees with the recent study reported by [33] but disagrees with the study carried out in the USA, which reported that approximately 10 % of patients are with colistin-resistant *A. baumannii* [15]. The Ab 333, Ab 976, and Ab 1289 were 90 % resistance and are CRAB, while Ab 150 and Ab 140 were 50 % and 40 % resistance, respectively. *Acinetobacter baumannii* identified in the LUTH clinic are all sensitive to colistin; however, 60% of the isolates were carbapenem-resistant *A. baumannii* (CRAB), as shown in Table 1. Thus, it becomes imperative for an urgent need for alternative antibiotic therapy. Phage therapy could offer one of the best applicable solutions to overwhelm the problem of antibiotic resistance of bacteria in Nigeria and the world. One of the striking merits of using phages over antibiotics in Nigeria is that phages are self-amplifying at the site of infection; so, phages can be given to patients in a single dose. Hence, the non-compliance of patients will not affect the success of the course of therapy. In this study, lytic and specific phages to MDR and XRD or CRAB *A. baumannii* (Ab 140, Ab 150, and Ab 333) were isolated from various environmental sources; from sewage and water drainage channel; this finding concurs with a report of other studies [20,34]. Interestingly, the environmental water from the drainage channel produces clear lytic phage plaques with high titers. It produced complete lysis several times without the emergence of phage-resistant mutants. Thus, the result contrasts the report that sewage was the best source to isolate highly lytic and specific phages of *A. baumannii* [35].

Morphologically, the phages (TJ 140, TJ 150, and TJ 333) formed clear round plagues with regular margins. The phages, TJ 140, and TJ 150 formed a clear plaque with a 2.0 to 4.0 mm diameter in 0.7 % TS agar plate and produce a halo-like appearance around the inhibition zone of lytic phage plaques after overnight incubation at 37 °C. This phenomenon indicates that these phages produce depolymerase, which degrades capsular exopolysaccharides-producing bacteria [36]. This result is in line with the report of [37]. However, TJ 333 phage formed a clear plaque with a 0.4 to 1.0 mm diameter in 0.7 % TSA plate and does not produce a halo-like appearance around the inhibition zone of lytic phages after overnight incubation at 37 °C. *In vitro* lytic analysis showed that the three phage isolates (TJ 333, TJ 140, and TJ 150) do not lyse XDR Ab 976 and Ab 1289. The phage cocktail is superior in activity than the single phage in lysing *A. baumannii* (Ab 140, Ab150, and Ab 333) isolates without developing resistant colonies. Such phage cocktails are good

candidates for preventing the emergence of phage-resistant mutants [38].

The evaluation of phage stability in environmental stress showed that they are relatively stable at high temperatures and more stable at alkaline pH than acidic pH. Every phage has an optimal pH for survival and biological activity. Thus, all the phages showed optimal viability at pH 6, with Phage TJ333 showing optimal stability at pH 5.0 and 6.0 and sharply reduced viability in acidic pH, this result concurs with the study as reported by [39]. When the pH was increased to 7.0, the phage titer dropped as in acidic pH. The phages TJ140 and TJ150 had optimum stability at pH 6.0 and 7.0, with TJ140 having over 50% viability across pH 4 to 7 showing better stability in an acidic environment than the other two phages, as seen in Figure 4.

All phages were stable across the temperatures tested in this study, with varying temperature effects observed. Phage TJ 333 exhibited maximum stability at 20 °C, while TJ 150 exhibited optimum stability at 37 °C physiological temperature, they were both stable across all temperatures (except 70 °C and 80 °C), with the least viability observed at 60 °C, the highest temperature at which growth was observed. However, Phage TJ 140 differed, it was relatively stable across temperatures 20 °C to 40 °C, and its optimum activity was at 60 °C. Incubation at more than 60°C for 1 hr was lethal to the phages, thus completely inactivating them. Such characteristics aligned with the results reported in previous studies [40,41], where phage viability ceased after exposure to temperatures above 60 °C.

From the pH and temperature stability data, phage infectivity remained intact at temperature and pH within and outside normal human physiological conditions. This should be a good characteristic for the phages intended for therapeutic applications.

The phages were evaluated for survival when stored for 12 months at 4 °C in SM buffer (pH 7.0). Figure 6 showed the rate at which the phages survive during storage. Phage TJ 140 survived better than others. This result showed that the pH of SM buffer favors TJ 140 more than TJ 150 and TJ 333. Therefore, storing phage for a long period requires that the phages are stored in the pH solution which supports their survival.

This study showed that the most reasonable condition observed for bacteriophage storage with minimal titer drop was 4 °C, which agrees with studies [42,43]

5. CONCLUSION

The findings in this study infer that *A. baumannii* in LUTH, Nigeria, are mostly MDR, XDR, and CRAB. Such high-rate multidrug-resistant bacteria demand novel methods to tackle this impending health risk in the community. Therefore, phage therapy assessed in this study was shown to be able to solve the problem of such hard-to-treat bacteria. The three phages isolated in this study were very lytic and stable across a broader range of temperatures, as observed in our peculiar environment, and could be useful for further studies into administering these phages systematically for the treatment of hospital-acquired infections caused by *Acinetobacter baumanii*.

6. RECOMMENDATIONS

We recommend that further work should be carried out in the characterization of these phages using phenotypic and molecular methods, as well as the development of phage cocktails to combat the resistant strains.

REFERENCES

1. Dhingra S, Rahman NAA, Peile E, Rahman M, Sartelli M, Hassali MA, et al. 2020 Microbial Resistance Movements: An Overview of Global Public Health Threats Posed by Antimicrobial Resistance, and How Best to Counter. Front Public Health. 4;8:531. https://doi.org/10.3389/fpubh.2020.535668
2. Lai CC, Chen SY, Ko WC, and Hsueh PR. 2021 Increased antimicrobial resistance during the COVID-19 pandemic. Int J Antimicrob Agents. 57(4):106324. https://doi.org/10.1016/j.ijantimicag.2021.106324
3. Serra-Burriel M, Keys M, Campillo-Artero C, Agodi A, Barchitta M, Gikas A, et al. 2020 Impact of multi-drug resistant bacteria on economic and clinical outcomes of healthcare-associated infections in adults: Systematic review and meta-analysis. PLoS One 15(1):e0227139. Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0227139
4. Shrivastava SR, Shrivastava PS, and Ramasamy J. 2018 World health organization releases global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Journal of Medical Society 32(1):76. Available from: https://www.jmedsoc.org/article.asp?issn=0972-4958;year=2018;volume=32;issue=1;spage=76;epage=77;aulast=Shrivastava
5. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. 2018 Discovery, research, and development of new antibiotics: the

WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 1;18(3):318–27. https://doi.org/10.1016/S1473-3099(17)30753-3

6. Centers for Disease control and Prevention. Antibiotic resistance threats in the United States, 2018. Atlanta, Georgia; 2019 Nov. Available from: https://stacks.cdc.gov/view/cdc/82532

7. World Health Organization. Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report. 2021. Available from: https://www.who.int/publications/i/item/9789240027336

8. Nasr P. 2020 Genetics, epidemiology, and clinical manifestations of multidrug-resistant Acinetobacter baumannii. J Hosp Infect. 104(1):4–11. Available from: https://pubmed.ncbi.nlm.nih.gov/31589900/

9. Moubareck CA and Halat DH. 2020 Insights into Acinetobacter baumannii: A Review of Microbiological, Virulence, and Resistance Traits in a Threatening Nosocomial Pathogen. Antibiotics. 9(3):119. Available from: https://www.mdpi.com/2079-6382/9/3/119/htm

10. Sarshar M, Behzadi P, Scribano D, Palamara AT, and Ambrosi C. 2021 baumannii: An Ancient Commensal with Weapons of a Pathogen. Pathogens 10(4):387. Available from: https://www.mdpi.com/2076-0817/10/4/387/htm

11. De Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, et al. 2020 Antimicrobial resistance in ESKAPE pathogens. Clin Microbiol Rev 33(3). Available from: https://journals.asm.org/doi/abs/10.1128/CMR.00181-19

12. Ogbolu DO, Alli OAT, Oluremi AS, Ogunjimi YT, Ojebode DI, Dada V, et al. 2020 Contribution of NDM and OXA-type carbapenemases to carbapenem resistance in clinical Acinetobacter baumannii from Nigeria. Infectious Diseases 52(9):644–50. https://doi.org/101080/2374423520201775881.

13. Higgins PG, Dammhayn C, Hackel M, and Seifert H. 2010 Global spread of carbapenem-resistant Acinetobacter baumannii. Journal of Antimicrobial Chemotherapy. 65(2):233–8. Available from: https://academic.oup.com/jac/article/65/2/233/685689

14. Nguyen M, and Joshi SG. 2021 Carbapenem resistance in Acinetobacter baumannii, and their importance in hospital-acquired infections: a scientific review. J Appl Microbiol. 131(6):2715–38. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/jam.15130

15. Qureshi ZA, Hittle LE, O'Hara JA, Rivera JI, Syed A, Shields RK, et al. 2015 Colistin-Resistant Acinetobacter baumannii: Beyond Carbapenem Resistance. Clinical Infectious Diseases 60(9):1295–303. Available from: https://academic.oup.com/cid/article/60/9/1295/404870

16. Doi Y, Murray GL, and Peleg AY. 2015 Acinetobacter baumannii: evolution of antimicrobial resistance-treatment options. Semin Respir Crit Care Med 36(1):85–98. Available from: https://pubmed.ncbi.nlm.nih.gov/25643273/

17. Hyman P. 2019 Phages for Phage Therapy: Isolation, Characterization, and Host Range Breadth. Pharmaceuticals 12(1):35. Available from: https://doi.org/10.3390/ph12010035

18. Abedon ST, García P, Mullany P, and Aminov R. 2017 Editorial: Phage Therapy: Past, Present and Future. Front Microbiol 8:981. Available from: https://doi.org/10.3389/fmicb.2017.00981

19. Bagińska N, Pichlak A, Górski A, and Jończyk-Matysiak E. 2019 Specific and Selective Bacteriophages in the Fight against Multidrug-resistant Acinetobacter baumannii. Virologica Sinica 34(4):347–57. Available from: https://link.springer.com/article/10.1007/s12250-019-00125-0

20. Kusradze I, Karumidze N, Rigvava S, Dvalidze T, Katsitadze M, Amiranashvili I, et al. 2016 Characterization and testing the efficiency of Acinetobacter baumannii phage vB-GEC_Ab-M-G7 as an antibacterial agent. Front Microbiol. 4;7(OCT):1590. https://doi.org/10.3389/fmicb.2016.01590

21. Grygorcewicz B, Roszak M, Golec P, Śleboda-taront D, Łubowska N, Górska M, et al. 2020 Antibiotics Act with vB_AbaP_AGC01 Phage against Acinetobacter baumannii in Human Heat-Inactivated Plasma Blood and Galleria mellonella Models. International Journal of Molecular Sciences 21(12):4390. Available from: https://www.mdpi.com/1422-0067/21/12/4390/htm

22. Wu N, Dai J, Guo M, Li J, Zhou X, Li F, et al. 2021 Pre-optimized phage therapy on secondary

Acinetobacter baumannii infection in four critical COVID-19 patients. Emerg Microbes Infect 10(1):612–8. Available from: https://www.tandfonline.com/doi/abs/10.1080/22221751.2021.1902754

23. Tan X, Chen H, Zhang M, Zhao Y, Jiang Y, Liu X, et al. 2021Clinical Experience of Personalized Phage Therapy Against Carbapenem-Resistant Acinetobacter baumannii Lung Infection in a Patient With Chronic Obstructive Pulmonary Disease. Front Cell Infect Microbiol. 26(11):50. https://doi.org/10.3389/fcimb.2021.631585

24. Garcia-Garrote F, and Cercenado E, Bouza E. 2000 Evaluation of a New System, VITEK 2, for Identification and Antimicrobial Susceptibility Testing of Enterococci. J Clin Microbiol. 38(6):2108–11. https://doi.org/10.1128/JCM.40.5.1681-1686.2002.

25. Hudzicki J. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol [Internet]. 2009. Available from: www.atcc.org

26. Enwuru N v., Gill JJ, Anttonen KP, Enwuru CA, Young R, Coker AO, et al. 2021 Isolation and characterization of novel phage (Podoviridae ϕParuNE1) and its efficacy against multi-drug-resistant Pseudomonas aeruginosa planktonic cells and biofilm. Beni Suef Univ J Basic Appl Sci. 2021 Dec 1;10(1). https://doi.org/10.1186/s43088-021-00137-4

27. Shende RK, Hirpurkar SD, Sannat C, Rawat N, and Pandey V. 2017 Isolation and characterization of bacteriophages with lytic activity against common bacterial pathogens. Vet World 10(8):973. https://doi.org/10.14202/vetworld.2017.973-978

28. Ozyurt S, Kostakoglu U, Yildiz IE, Erturk A, Sonmez E, Sahin U, et al. 2020 Investigation of the clonal associations in Acinetobacter Baumannii strains isolated from the respiratory samples of patients in a tertiary research hospital. Niger J Clin Pract. 1;23(8):1155–62. https://doi.org/10.4103/njcp.njcp549_18

29. Xie R, Zhang XD, Zhao Q, Peng B, and Zheng J. 2018Analysis of global prevalence of antibiotic resistance in Acinetobacter baumannii infections disclosed a faster increase in OECD countries. Emerg Microbes Infect 7(1). Available from: https://www.tandfonline.com/doi/abs/10.1038/s41426-018-0038-9

30. Rangel K, Chagas TPG, and De-Simone SG. 2021 Acinetobacter baumannii Infections in Times of COVID-19 Pandemic. Pathogens 10(8):1006. Available from: https://www.mdpi.com/2076-0817/10/8/1006/htm

31. Bashir A, Garba I, Aliero AA, Kibiya A, Abubakar MH, Ntulume I, et al. 2019 Superbugs-related prolonged admissions in three tertiary hospitals, Kano State, Nigeria. Pan Afr Med J. 32:166. https://doi.org/10.11604/pamj.2019.32.166.18481

32. Odewale G, Adefioye OJ, Ojo J, Adewumi FA, Olowe OA. 2016 Multidrug resistance of Acinetobacter baumannii in Ladoke Akintola University Teaching Hospital, Osogbo, Nigeria. Eur J Microbiol Immunol (Bp). 6(3):238–43. https://doi.org/10.1556/1886.2015.00018.

33. Uwingabiye J, Frikh M, Lemnouer A, Bssaibis F, Belefquih B, Maleb A, et al. 2016 Acinetobacter infections prevalence and frequency of the antibiotics resistance: comparative study of intensive care units versus other hospital units. PAMJ 23(191). Available from: https://www.panafrican-med-journal.com/content/article/23/191/full

34. Cha K, Oh HK, Jang JY, Jo Y, Kim WK, Ha GU, et al. 2018 Characterization of two novel bacteriophages infecting multidrug-resistant (MDR) Acinetobacter baumannii and evaluation of their therapeutic efficacy in vivo. Front Microbiol 10(9)696. https://doi.org/10.3389/fmicb.2018.00696

35. Łobocka M, Dąbrowska K, and Górski A. 2021 Engineered Bacteriophage Therapeutics: Rationale, Challenges and Future. BioDrugs 35(3):255–80. Available from: https://link.springer.com/article/10.1007/s40259-021-00480-z

36. Hernandez-Morales AC, Lessor LL, Wood TL, Migl D, Mijalis EM, Cahill J, et al. 2018 Genomic and Biochemical Characterization of Acinetobacter Podophage Petty Reveals a Novel Lysis Mechanism and Tail-Associated Depolymerase Activity. J Virol 92(6). Available from: https://pubmed.ncbi.nlm.nih.gov/29298884/

37. Fang Q, and Zong Z. 2022 Lytic Phages against ST11 K47 Carbapenem-Resistant Klebsiella pneumoniae and the Corresponding Phage Resistance Mechanisms. mSphere 7(2). https://journals.asm.org/doi/10.1128/msphere.00080-22

38. Jasim HN, Hafidh RR, and Abdulamir AS. 2018 Formation of therapeutic phage cocktail and endolysin to highly study multi-drug resistant Acinetobacter

baumannii: In vitro and in vivo. Iran J Basic Med Sci. 1;21(11):1100–8. https://doi.org/10.22038/IJBMS.2018.27307.6665.

39. Wintachai P, Surachat K, and Singkhamanan K. 2022 Isolation and Characterization of a Novel Autographiviridae Phage and Its Combined Effect with Tigecycline in Controlling Multidrug-Resistant Acinetobacter baumannii-Associated Skin and Soft Tissue Infections. Viruses. 1;14(2). https://doi.org/10.3390/v14020194

40. Wintachai P, Naknaen A, Pomwised R, Voravuthikunchai SP, and Smith DR. 2019 Isolation and characterization of Siphoviridae phage infecting extensively drug-resistant Acinetobacter baumannii and evaluation of therapeutic efficacy in vitro and in vivo. J Med Microbiol 68(7):1096–108. Available from: https://www.microbiologyresearch.org/content/journal/jmm/10.1099/jmm.0.001002

41. Jeon J, Park JH, and Yong D. Efficacy of bacteriophage treatment against carbapenem-resistant Acinetobacter baumannii in Galleria mellonella larvae and a mouse model of acute pneumonia. BMC Microbiol 19(1):1–14. Available from: https://bmcmicrobiol.biomedcentral.com/articles/10.1186/s12866-019-1443-5

42. Miller H. 1987 Practical aspects of preparing phage and plasmid DNA: growth, maintenance, and storage of bacteria and bacteriophage. Methods Enzymol [Internet]. 152(C):145–70. https://pubmed.ncbi.nlm.nih.gov/2958676/

43. Alvi IA, Asif M, Tabassum R, Abbas Z, and Ur Rehman S. 2018 Storage of bacteriophages at 4°C leads to no loss in their titer after one year. Pak J Zool. Dec 1;50(6):2395–8. https://doi.org/10.17582/journal.pjz/2018.50.6.sc8