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Potential application of bacteriophage to control carbapenem-resistant *Acinetobacter baumannii* isolated from inanimate hospital surfaces

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ABSTRACT

Acinetobacter baumannii is one of the most crucial pathogens causing nosocomial infections in hospitals due to its simplicity of extraordinarily evolving multi-drug resistance. This study aimed to isolate carbapenem-resistant Acinetobacter baumannii (CRAB) from inanimate hospital surfaces and evaluate the efficacy of a newly isolated phage in combating these isolates. A selective and differential chromogenic culture medium, CHROMagar™ Acinetobacter was used for selective isolation of CRAB, followed by MALDI-TOF to identify the isolate, the Vitek 2 system to determine their antibiograms, and PCR to detect the carbapenemase genes. Furthermore, an *Acinetobacter*-specific phage was characterized and isolated from sewage using the enrichment technique. Results revealed the isolation of 38 carbapenem-resistant *Acinetobacter* spp. Of these, 30 isolates were identified as *A. baumannii* and their antibiograms showed high levels of resistance against various categories of antibiotics. All the isolates investigated harbor two carbapenemase genes, blaOXA-51 and blaOXA-23. Moreover, the isolated phage ϕAB1.1 exhibited high specificity to various isolates of *A. baumannii* and did not infect other bacterial species. The phage was stable at temperatures up to 60 °C and over the pH range between 5.0 to 11.0. These findings suggested the potential application of ϕAB1.1 as a promising agent to control CRAB.

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Introduction

Acinetobacter baumannii is recognized as one of the most crucial pathogens when it comes to nosocomial infections in hospitals throughout the world due to its simplicity of extraordinarily evolving multi-drug resistance (Moubareck & Halat, 2020). Recently, this bacterium has been genetically modified to oppose the action of the last-resort carbapenem class of antibiotics and has been classified as carbapenem-resistant *A. baumannii* (CRAB) (Nguyen & Joshi, 2021; Ijaz *et al.*, 2024).

Moreover, the ability of *A. baumannii* to contaminate and persist in hospital environments is a critical issue, especially its ability to survive in environmental conditions (Ahuatzin-Flores *et al.*, 2024). In hospitals, the main sources of infections are the clinical patients and healthcare workers, but the role of the hospital environment, particularly the inanimate surfaces, as a source and reservoir of transmission is a concern to devise effective control measures (Patelarou *et al.*, 2022; Abarca-Coloma *et al.*, 2024). It has been reported that *A.*

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baumannii has a remarkable ability to become established in nosocomial settings through colonization of patients and contamination of the patients' microenvironment, especially inpatient care equipment and healthcare personnel therefore *A baumannii* is one of the most persistent bacteria known on inanimate surfaces (Meschiari *et al.,* 2021). Furthermore, their ability to survive for an extended period on inanimate surfaces, giving rise to outbreaks in hospital departments with routine use of life-supporting medical devices, especially in intensive care units (Ababneh *et al.,* 2022).

Interest in alternatives to conventional antibiotics has been raised due to the continuous emergence of multidrug-resistant bacteria. Bacteriophages (phages) are viruses that infect bacteria and are ubiquitous in the environment. Phages can specifically recognize and kill their host bacterial cells (Raza *et al.,* 2021). Since the discovery of phages, the idea of using phages to infect and kill bacteria for medical purposes has been around. Phage therapy is attractive as an alternative option to reduce antibiotic-related side effects without disrupting the balance of the microbiome in immunocompromised patients *(Puccetti et al.,* 2020). Phages can recognize specific peptidoglycan structures within specific outer membrane proteins, lipopolysaccharides, and capsule polysaccharides, and can attack specific peptidoglycan structures (Grabowski et al. 2021). There are previous investigations on several *Acinetobacter phages* isolated from different channels of aquatic environments to control several *A. baumannii* strains (Sisakhtpour *et al.,* 2022; Hailemichael *et al.,* 2023). This study addresses the isolation and characterization of CRAB from inanimate hospital surfaces and evaluates the efficacy of an *Acinetobacter*-specific phage isolated from sewage in combating CRAB.

Materials and Methods

Isolation and Identification of Carbapenem-Resistant Acinetobacter spp.

Swab samples were collected from exposed inanimate hospital surfaces such as doorknobs, drawer handles, nurse call buttons, bedside tables, bed rails, sinks, and faucets at a surgical intensive care unit in our previous study (Fahmy *et al.,* 2024). The collected swabs were streaked on CHROMagar™ *Acinetobacter* plates (CHROMagar, France), and the inoculated plates were incubated at 35-37 °C for 18-24 h. Subsequently, the presumptive carbapenem-resistant colonies were picked and identified by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry using a Microflex LT device (Bruker Daltonics, Bremen, Germany) and analyzed by the Biotyper software (Farahat, 2020). The identification of CRAB isolates was further confirmed based on their 16S rRNA gene sequences as described elsewhere (Kamel *et al.,* 2016; Farahat, 2019).

Antimicrobial Susceptibility Testing

All CRAB isolates were subjected to antimicrobial susceptibility testing using the VITEK 2® automated equipment (bioMérieux, France) with Vitek2® Gram Negative Susceptibility cards (AST-GN67) according to manufacturer's guidelines.

Detection of Carbapenemase Genes

Template DNA for polymerase chain reaction (PCR) was extracted by boiling method as described previously (Ghaith *et al.,* 2019). Briefly, 2-3 colonies of each bacterial isolate were suspended in 100 µL of PCR-grade water, boiled for 10 min, snap-cooled, and centrifuged at 14000 rpm for 5 min. Subsequently, multiplex PCR was performed to detect $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-58-like}}$ carbapenemases as described previously (Woodford *et al.,* 2006) using the bla_{OXA}-specific primers (Table 1).

Isolation of Bacteriophages

Bacteriophages were isolated from sewage samples using the enrichment technique (Topka *et al.,* 2019). The sewage samples were centrifuged for 20 min at 6000 rpm, and the supernatant was filtered through membrane filters with 0.22 µm pore size. Subsequently, the filtrate was combined with an equivalent volume of sterile 2X Brain Heart Infusion (BHI) broth in 250 mL Erlenmeyer flasks. The flask was inoculated with the highest resistant CRAB isolate and incubated with shaking (120 rpm) at 37 °C for 24 h. After centrifuging the culture for 10 min at 6000 rpm, the cells were discarded, and the supernatant was filtered through a 0.22 µm membrane filter. The filtrates were then examined for *Acinetobacter*-specific phages.

Spot Test

The spot test using the double agar overlay technique was conducted to detect the presence of phages in the enriched filtrates (Daubie *et al.,* 2022). Briefly, 100 µL of an overnight culture of CRAB isolate was added to a semi-solid BHI medium and overlayed solid BHI agar plate. After solidifying, 10 µL of the prepared enriched filtrates were spotted on the bacterial lawns and left to dry. After incubation overnight at 37 ºC, the plates were checked for the formation of the lysed zones.

Determination of Phage Titer

The phage titer was determined via plaque assay (Jurczak-Kurek *et al.,* 2016). In brief, ten-fold serial dilutions of phage filtrate were prepared using sterile saline (8.5 g NaCl/L) as diluent. Then, 100 µL aliquots of overnight CRAB culture were mixed with equal volumes

of the diluted phage suspensions and added to molten semi-solid BHI agar. The mixtures were poured onto solid BHI agar plates. After solidifying, the plates were incubated overnight at 37 ºC to enumerate plaques. Subsequently, plaques were counted to determine the phage titer [expressed as plaques forming units per mL (PFU/mL)]. The titer of the phage was expressed as follows:

Titer = number of plaques x 10 reciprocal of dilution.

Table 1. Primers used in the multiplex PCR

Primer[a]	Sequence (5′–3′)	Gene	Product size (bp)
OXA23-F	GATCGGATTGGAGAACCAGA	*bla*$_{\text{OXA-23-like}}$	501
OXA23-R	ATTTCTGACCGCATTTCCAT		
OXA24-F	GGTTAG TTGGCCCCCTTAAA	*bla*$_{\text{OXA-24-like}}$	246
OXA24-R	AGTTGAGCGAAAAGGGGATT		
OXA51-F	TAATGCTTTGATCGGCCT TG	*bla*$_{\text{OXA-51-like}}$	353
OXA51-R	TGGATTGCACTTCATCTTGG		
OXA58-F	AAGTATTGGGGCTTGTGCTG	*bla*$_{\text{OXA-58-like}}$	599
OXA58-R	CCCCTCTGCGCTCTACATAC		

[a]F, sense primer; R, antisense primer.

Purification of Bacteriophages

A single plaque was picked and added to 5.0 mL of BHI broth inoculated with 100 µL of overnight CRAB culture. After incubation at 37 ºC for 24 h in a shaking incubator, the phage-host mixture was centrifuged at 6000 rpm for 10 min, and the supernatant was filtrated through a 0.45µm-pore size Millipore filter to get rid of the bacterial cells. After repeating the process five times, the final supernatant was used as a source of phage (Abdel-Haliem & Askora, 2013).

Transmission Electron Microscopy

To examine the morphology of the phage's virion, the phage plaques developed on the agar plate were washed off and filtered through a membrane filter with a pore size of 0.22 µm. The transmission electron microscopy (TEM) of the purified phage was performed employing the JEM-1400 TEM equipment (Jeol, Japan) operated at 80 kV, by using negative staining with 2% potassium phosphotungstate, as described previously (Peng *et al.*, 2020).

Host Range of Isolated Phage

The host range of the isolated phage was tested against three clinical carbapenem-resistant *A. baumannii* isolates, *A. baumannii* (ATCC 19606), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* subsp. *enterica* (ATCC 14028), *Staphylococcus aureus* subsp. *aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), and *Bacillus subtilis* (ATCC 6633). Practically, 50 µL of overnight culture of each assessed bacterial isolate was added to 3 mL of molten semi-solid BHI medium and the mixture was then overlaid on BHI agar plates. Afterward, 10 µL of phage suspension was spotted on the lawns of each tested bacterial isolate, followed by overnight incubation at 37 °C. Subsequently, plates were observed, and the presence of lytic zones was interpreted as evidence of bacterial susceptibility to phage-mediated lysis (Amankwah *et al.*, 2022).

Physical Stability of the Phage

The thermal stability of the purified phage was determined by subjecting newly prepared phage suspensions (10^{13} PFU/mL) to various temperatures ranging from 30 to 80 °C for 30 min. Subsequently, the titers of the phage were determined. The pH tolerance of the isolated phage was assessed by incubating the phage in buffers with various pH values (ranging from 3 to 12) for 30 min at 28 °C. Afterward, the titers of the phage were determined by the double-agar overlay method (Li *et al.*, 2022).

Results

In this study, 38 presumptive carbapenem-resistant *Acinetobacter* spp. isolates were recovered from various inanimate hospital surfaces using CHROMagar™ *Acinetobacter* plates. Based on MALDI-TOF analysis, 30 isolates were identified as *A. baumannii*. Accordingly, the confirmed CRAB isolates were selected for further investigations. The identification of CRAB was further confirmed by amplification and sequencing of the 16S rRNA gene. BLAST analysis revealed that the 16S rRNA gene shared 100% identity of *A. baumannii*, confirming the results obtained by MALDI-TOF. The sequence of the

16S rRNA gene was submitted to the GeneBank, the accession number PQ014154 was assigned, and the Neighbor-Joining phylogenetic tree of CRAB was constructed (Fig. 1).

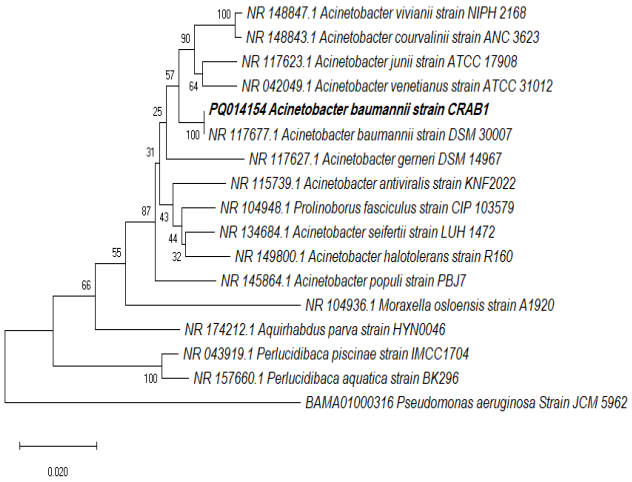

Fig. 1. Phylogenetic tree showing the relationship between *A. baumannii* and the most closely related species in the context of the 16S rRNA gene sequences using the Neighbor-Joining method.

In this study, CRAB isolates exhibited high levels of antibiotic resistance against various categories of antibiotics. In this regard, 100% of CRAB isolates were resistant to carbapenem agents (ertapenem and imipenem), ampicillin, cefazolin, ceftazidime, and ceftriaxone (Table 2). However, the investigated CRAB isolates were more sensitive to aminoglycosides and fluoroquinolones. Regarding aminoglycosides, 6/30 isolates were susceptible to amikacin, 5/30 were susceptible to gentamicin, and 4/30 were susceptible to tobramycin. With respect to fluoroquinolones, 7/30 isolates were susceptible to cibrofloxacin and 4/30 were susceptible to levofloxacin. Genotypic screening of the CRAB isolates revealed that all isolates (100%) harbor both $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-23}}$ genes (Fig. 2). On the contrary, all the isolates were negative for the other two carbapenemase genes ($bla_{\text{OXA-24}}$ and $bla_{\text{OXA-58}}$).

Sewage samples were collected as a potential source for isolating bacteriophages against CRAB isolates using the enrichment technique. The results showed the formation of clear lytic zones on lawns of CRAB isolates, indicating the successful recovery of *Acinetobacter*-specific phage designated ϕAB1.1. The plaques produced by the isolated phage appeared clear and circular with an average diameter of 4 mm (Fig. 3). TEM analysis showed that ϕAB1.1 has a capsid of 55 nm in diameter and non-contractile tail (Fig. 4). The host range of phages ϕAB1.1 was assessed with clinical CRAB isolates and *A. baumannii* ATCC 19606 as well as strains of other Gram-negative and Gram-positive species. The results showed that ϕAB1.1 produced clear plaques on bacterial lawns of all investigated *A. baumannii*. On the other hand, no cross-sensitivity to non-*A. baumannii* strains, such as *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *S. enterica* subsp. *enterica* ATCC 14028, *S. aureus* subsp. *aureus* ATCC 6538, *S. epidermidis* ATCC 12228, and *B. subtilis* ATCC 6633 was detected. The temperature and pH stability evaluations revealed that phage ϕAB1.1 was relatively stable at temperatures up to 60 °C and at pH values between 5.0 and 11.0. However, the phage could not withstand temperatures of 70 °C and pH of 4.0 and 12.0.

Discussion

Antibiotic resistance has become an indispensably alarming menace that poses a serious global threat of growing concern to human, animal, and environmental health (Aslam *et al.,* 2018; Fadl *et al.,* 2022; Akram *et al.,* 2023). Particularly, *A. baumannii* is one of the most common clinically important gram-negative MDR bacteria that dominate as a major crisis for hospitals due to its fast spread and noticeable ability to acquire resistance determinants against antimicrobial agents (Lam & Hamidian, 2024; Shi *et al.,* 2024). Recently, essential oils, natural products, nanomaterials, and phages have gained attention as potential alternatives to antibiotics (Ganić *et al.,* 2022; Abd El-Ghany *et al.,* 2023; Ali *et al.,* 2024; Subramanian, 2024; Sayed *et al.,* 2024). In this study, we addressed the isolation and characterization of CRAB from inanimate hospital surfaces and the efficacy of the newly isolated phage ϕAB1.1 as a potential agent for combating these superbugs. Herein, we used the CHROMagar™ *Acinetobacter* plates for rapid and efficient isolation of carbapenem-resistant *Acinetobacter* spp. from inanimate hospital surfaces. Recently, the CHROMagar™ *Acinetobacter* was recommended for isolation and phenotypic detection of carbapenem-resistant *Acinetobacter* spp. from various sources (Hubeny *et al.,* 2022; Yusuf *et al.,* 2023; Lellouche *et al.,* 2024). In this study, the recovered isolates were identified by MALDI-TOF as *A. baumannii* and designated CRAB. These findings are in harmony with those reporting that healthcare equipment and inanimate hospital surfaces are reservoirs of MDR *A. baumannii* CRAB (Kuczewski *et al.,* 2022; Azaiez *et al.,* 2023; Osman *et al.,* 2024). In this study we observed high resistance levels of most CRAB isolates to more than three categories of antibiotics, classifying these isolates as MRD *A. baumannii*. These findings agree with previous studies that alarming the

rapid emergence of antibiotic resistance among *A. baumannii* and the global spread of MRD *A. baumannii* strains (Kyriakidis *et al.,* 2021; Ibrahim *et al.,* 2021; Cogliati Dezza *et al.,* 2023; Abbasi *et al.,* 2023; Xiong *et al.,* 2023). In this investigation, molecular characterization of CRAB revealed that all isolates harbor *bla*$_{OXA-51}$ and *bla*$_{OXA-23}$ genes. These results are consistent with the previous study reporting that all CRAB isolates harbored intrinsic *bla*$_{OXA-51-like}$ class D carbapenemases (Rao *et al.,* 2020). Both, intrinsic and acquired class D carbapenemases like *bla*$_{OXA-51}$ and *bla*$_{OXA-23}$, are the most prevalent in CRAB worldwide (Garciglia-Mercado *et al.,* 2021; Zhu *et al.,* 2022; Raddaoui *et al.,* 2024). These results indicate the genetic basis for the resistance.

In this research, we isolated an *Acinetobacter*-specific phage ϕAB1.1 from sewage and evaluated its efficacy in combating CRAB. It has been suggested that sewage contains abundant phages that survive on drug-resistant bacteria and can serve as a preferred resource for phage isolations (Du *et al.,* 2021). Such plaque morphology indicated that ϕAB1.1 is a lytic bacteriophage. In a recent similar study, the *Acinetobacter* phage "Abgy202141" isolated from underground sewage from residential areas

Antimicrobial Categories	Antimicrobial Agents	Number of Isolates		
		S	I	R
Aminoglycosides	Amikacin (AN)	6/30	0/30	24/30
	Gentamicin (GM)	5/30	0/30	25/30
	Tobramycin (TM)	4/30	6/30	20/30
Penicillin	Ampicillin (AM)	0/30	0/30	30/30
Penicillin with β-lactamase inhibitor	Piperacillin/tazobactam (TZP)	2/30	1/30	27/30
	Ampicillin/sulbactam (SAM)	2/30	0/30	28/30
First-generation cephalosporin	Cefazolin (CZ)	0/30	0/30	30/30
Third-generation cephalosporin	Ceftazidime (CAZ)	0/30	0/30	0/30
	Ceftriaxone (CRO)	0/30	0/30	30/30
Fourth-generation cephalosporin	Cefepime (FEP)	3/30	0/30	27/30
Fluoroquinolones	Ciprofloxacin (CIP)	7/30	5/30	18/30
	Levofloxacin (LEV)	4/30	3/30	23/30
Carbapenem agents	Ertapenem (ETP)	0/30	0/30	30/30
	Imipenem (IPM)	0/30	0/30	30/30
Nitrofuran derivative	Nitrofurantoin (FT)	1/30	0/30	29/30
Diaminopyrimidine with sulfonamide	Trimethoprim/Sulfamethoxazole (SXT)	1/30	2/30	27/30

R: Resistant, I: Intermediate, S: Sensitive

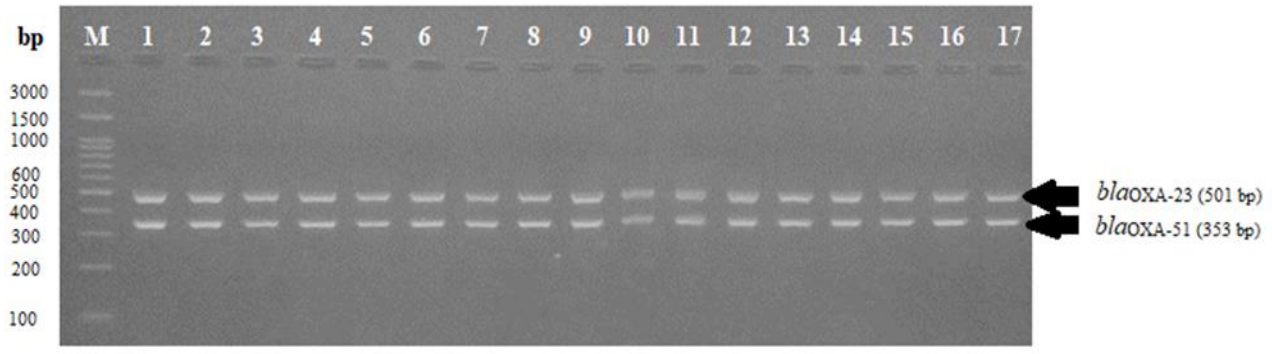

Fig. 2. Detection of carbapenemase genes in CRAB isolates by Multiplex PCR. Lane M, DNA ladder; lanes 1-17, PCR products of 17 CRAB isolates showing the presence of *bla*$_{OXA-23}$ (501 bp) and *bla*$_{OXA-51}$ (353 bp) genes and absence of *bla*$_{OXA-24}$ and *bla*$_{OXA-58}$ genes.

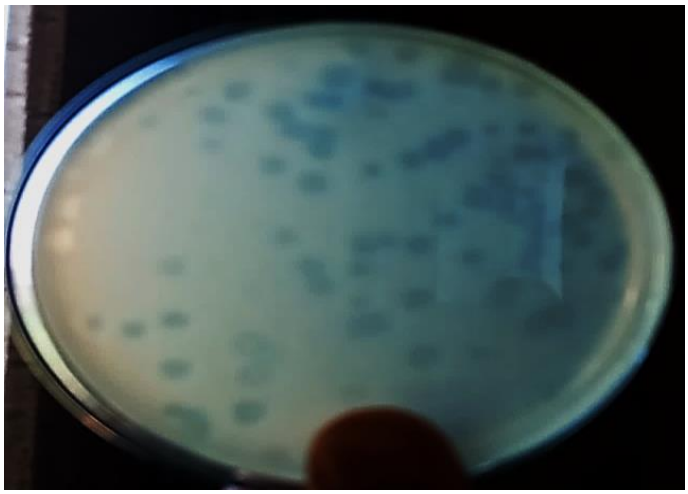

Fig. 3. Plaque morphology of the *Acienetobacter* phage ϕAB1.1.

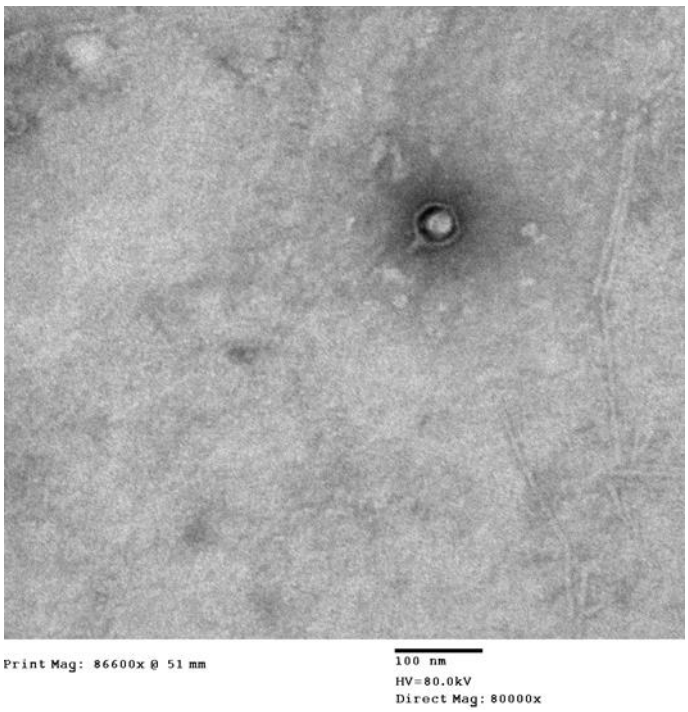

Fig. 4. TEM micrograph showing the *Acienetobacter* phage ϕAB1.1.

exhibited the ability to produce transparent circular plaques in the double-agar plaque assay (Tian *et al.*, 2024). It is worth mentioning that the bacteriophage ϕAB1.1 isolated in this study can lyse various *A. baumannii* isolates but did not exhibit the ability to infect strains of the remaining species. This selectivity could be because the isolation procedure was based on the one-host enrichment method in which a raw sewage sample was mixed with CRAB culture to obtain the lysate (Topka *et al.*, 2019). Likewise, *A. baumannii* isolates were lysed by bacteriophage "vB-AbauM-Arak1" while no clear plaques were observed in other assessed bacterial species indicating that phage vB-AbauM-Arak1 was specific to *A. baumannii* (Ghaznavi-Rad *et al.*, 2022). It has been reported that *A. baumannii*-specific bacteriophages (BPABΦ1) showed a narrow host range, suggesting that phages obtained from multidrug-resistant *A. baumannii* have narrow host-specificity (Dehari *et al.*, 2023).

Phage's physical tolerance to environmental conditions is attributed to its thermal and acid-base stabilities, which are crucial characteristics. In this study, the ϕAB1.1 kept its activity at temperatures up to 60 °C, while at 70 °C the lytic spot vanished suggesting that the thermal inactivation point was 70 °C. These findings concord with those reported on the *Acinetobacter*-specific phage (Ab_WF01) which showed the highest viability at 25 °C, and its stability decreased by increasing the temperature, until completely no viability when the temperature reached 70 °C (Wang *et al.*, 2024).

Conclusion

Inanimate hospital surfaces are reservoirs of CRAB with high levels of resistance to various categories of antibiotics due to the acquisition of carbapenemase genes. All the investigated CRAB isolates in this study were positive for $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-23}}$ genes, confirming the phenotypic detection of carbapenem resistance. The *Acinetobacter*-specific phage ϕAB1.1 isolated from sewage had high specificity to *A. baumannii* isolates, and could not infect other bacterial species, promising stability in wide ranges of temperatures and pH values. These results shed light on the potential use of ϕAB1.1 for combating CRAB on inanimate hospital surfaces to diminish hospital-acquired infections with CRAB.

Conflict of interest

The authors declare that they have no conflict of interest.

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