FAGOAQUA: Characterization of a Lytic Phage from Urban Wastewater against Multidrug Resistant Bacteria

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ABSTRACT:

Antimicrobial resistance is a critical global health threat, directly causing 1.27 million deaths in 2019 (Murray et al., 2022). Wastewater treatment plants (WWTPs) act as significant hotspots where multidrug-resistant (MDR) bacteria persist and spread into the environment (Godinho, Lage, & Quinteira, 2024). At the same time, these systems represent dynamic environments that promote high bacteriophage diversity and host–phage interactions. The FAGOAQUA project aims to isolate and characterize MDR bacterial strains and their associated lytic phages from wastewater. Experimental results confirmed the isolation of several MDR strains, specifically Raoultella ornithinolytica, Citrobacter freundii, and Proteus mirabilis, all of which exhibited different resistance levels to multiple antimicrobial classes. Concurrently, a lytic bacteriophage was isolated and characterized, demonstrating lytic activity against one of these strains. Together, these results highlight the value of wastewater environments as reservoirs for the identification of phages with potential applicability in the targeted control of antibiotic-resistant bacteria.

Keywords: Bacteriophage; MDR; WWTP

INTRODUCTION:

Antimicrobial resistance (AMR), and particularly bacterial resistance to antibiotics, has emerged as one of the most critical global public health challenges of the 21st century. In 2019, antibiotic-resistant infections were estimated to be directly responsible for 1.27 million deaths worldwide, underscoring the substantial global burden of AMR; global projections further indicate that this figure could rise to up to 10 million deaths annually by 2050 (Murray et al., 2022).

Although WWTPs are significant contributors to the global spread of AMR by allowing MDR bacteria to persist and migrate into the environment (Godinho, Lage, & Quinteira, 2024), they also hold the key to a promising therapeutic future. These plants function as the ideal ecosystem for bacteriophages, acting as rich biological 'mines' where phages naturally evolve to infect resistant strains. By shifting our perspective, these focal points of resistance can be transformed into essential hubs for discovering new lytic agents, offering a hopeful alternative for overcoming the limitations of modern antibiotic therapy. Beyond their ecological role, these naturally evolved phages also stand as powerful therapeutic tools, offering targeted, safe, and effective options for treating multidrug-resistant infections where conventional antibiotics fail (Alaaeddine et al., 2025)

FAGOAQUA aims to isolate and characterize MDR bacterial strains along with their corresponding lytic phages found in wastewater systems. By revaluing these environments as a strategic source for new lytic agents, the project offers a hopeful and sustainable biotechnological approach to combat the rising challenge of antibiotic resistance.

RESULTS:

Isolation, Identification, and Characterization of AMR bacterial strains

Four antibiotic-resistant bacterial strains (N1, A1, A2, and A3) were successfully isolated from wastewater samples obtained from Basque WWTPs. All isolates were identified as Gram-negative bacilli. Pathogenicity testing revealed that strain N1 incorporated Congo Red dye, suggesting a capacity for biofilm formation and potential invasiveness (Berkhoff & Vinal, 1986; Cho et al., 2022).

16S rRNA gene sequencing allowed the identification of the four isolates: A1 was identified as Raoultella ornithinolytica, N1 as Citrobacter freundii, and both A2 and A3 as Proteus mirabilis. A critical finding across all four extractions was the presence of plasmids, which is biologically consistent as these extrachromosomal elements are usually vehicles for the AMR genes (Bennett, 2008). The phylogenetic relationships were established using the NGPhylogeny.fr platform (Lemoine et al, 2019) , resulting in a phylogram that compares these isolates with 16 other bacterial species to visualize their evolutionary proximity, as shown in Figure 1.

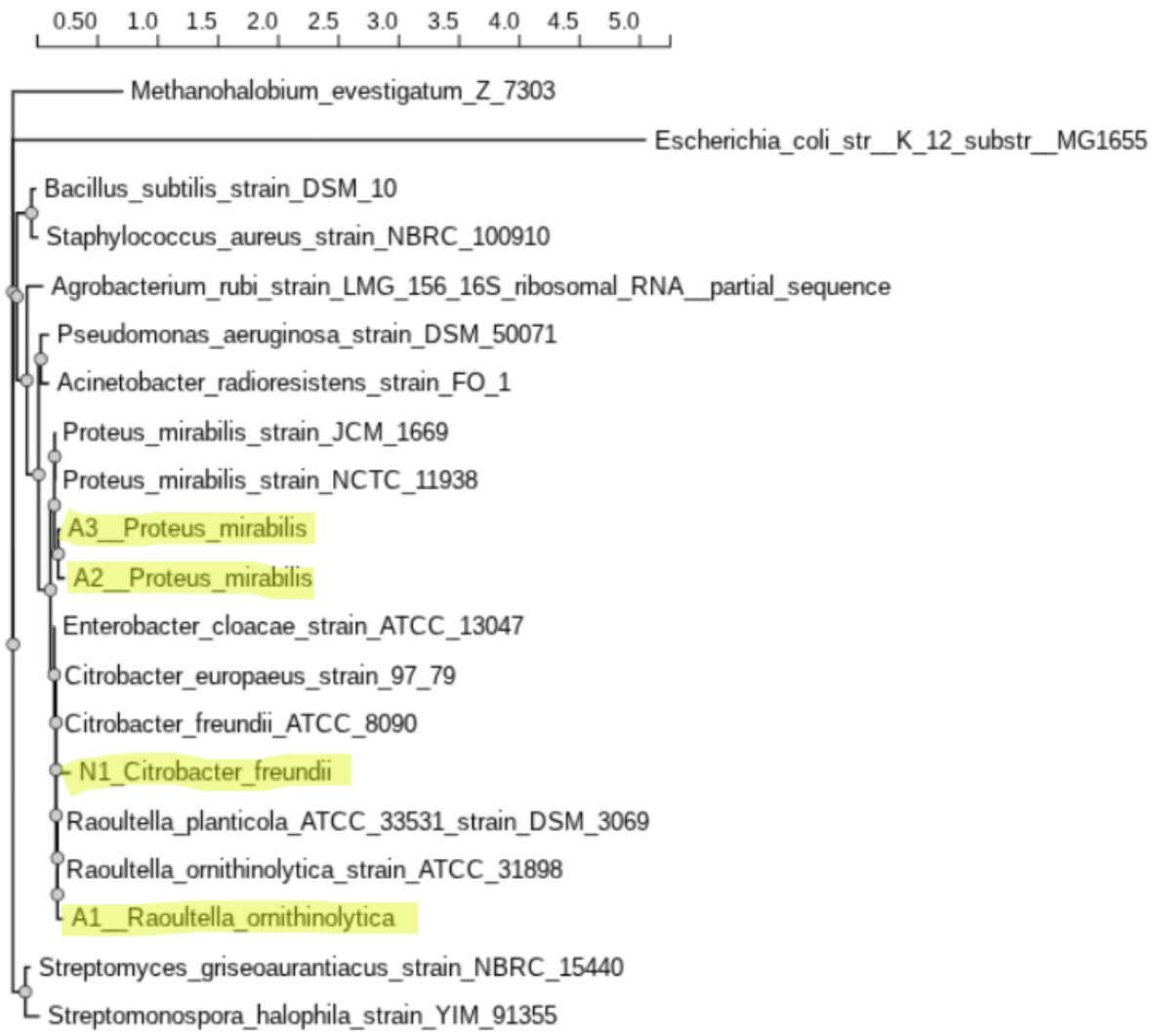

Figure 1 . Linear phylogram of the isolated bacterial strains (A1, N1, A2, and A3) and related species, constructed using the PhyML algorithm based on 16S rRNA gene sequences.

Antimicrobial susceptibility testing by disk diffusion showed that all four isolates were commonly resistant to erythromycin and clindamycin (Figure 2). Strain A1 (*Raoultella ornithinolytica*) did not exhibit additional resistance but showed intermediate sensitivity to amoxicillin/clavulanic acid. In contrast, strain N1 (*Citrobacter freundii*) and the *Proteus mirabilis* strains A2 and A3 displayed an extended resistance profile including gentamicin and tetracycline. Differences among these three strains were limited to intermediate responses: N1 to amoxicillin/clavulanic acid, A2 to ciprofloxacin, and A3 to ceftazidime.

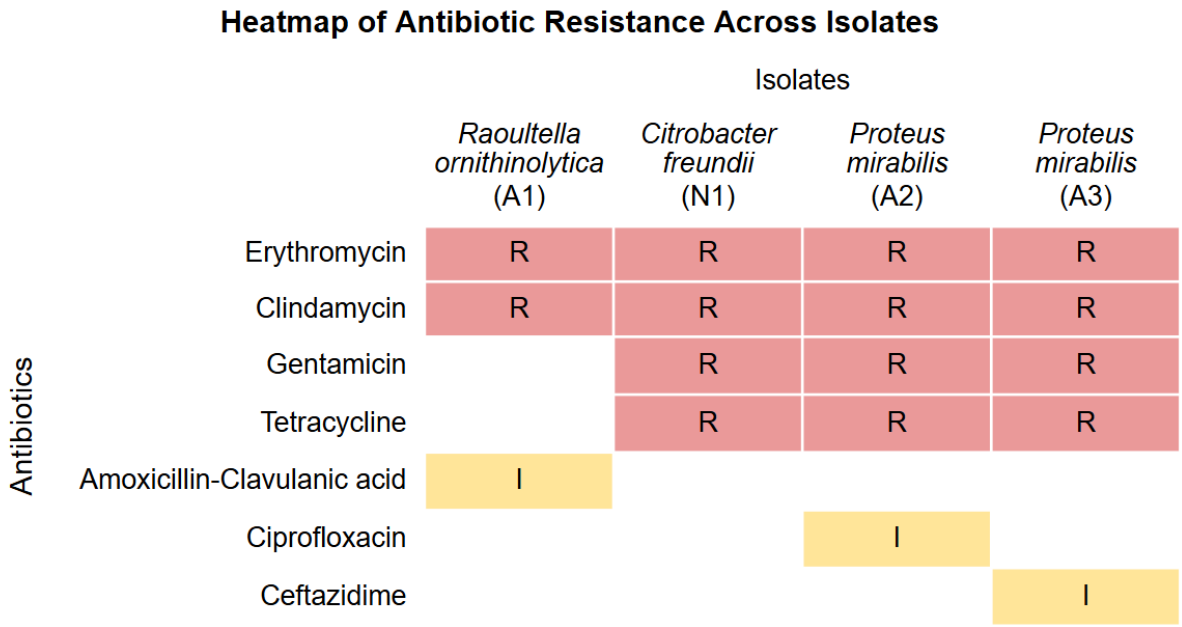

Figure 2 Heatmap of antibiotic susceptibility profiles for the four isolates. Columns correspond to isolates (A1, N1, A2, A3), rows to antibiotics tested. Colors indicate susceptibility categories: red = resistant (R), yellow = intermediate (I).

Isolation and Characterization of Bacteriophage GA-A1-1

For the MDR isolate A1 (*Raoultella ornithinolytica*), a specific phage designated as GA-A1-1 was successfully recovered and isolated from one of the WWTPs. Plaque assay results yielded a phage titer of 1.8 ·10^5 PFU/mL (Figure 3).

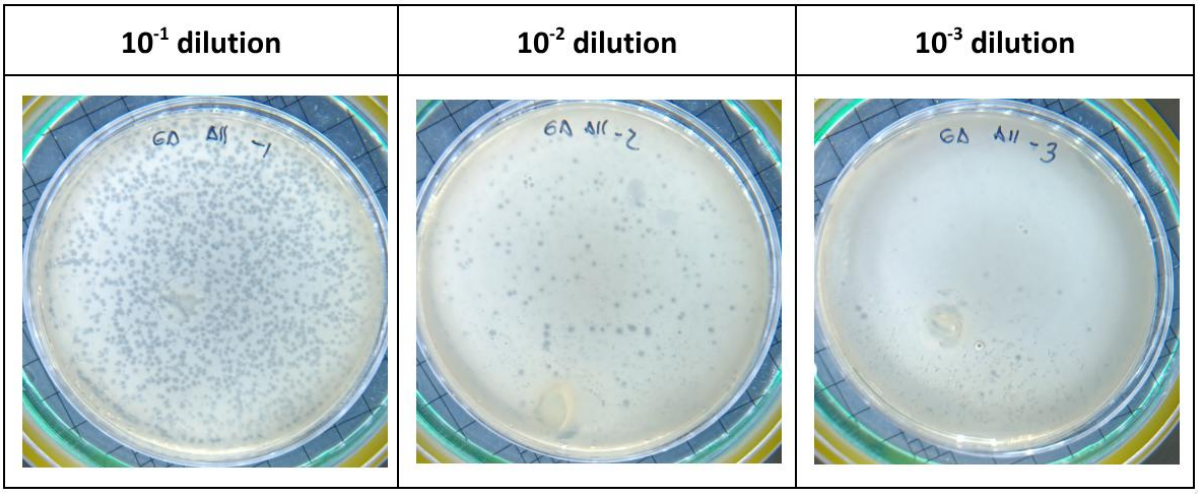

Figure 3 Double-layer agar phage titration assay of isolate GA-A1-1 showing lytic plaques on ampicillin-supplemented LB agar medium. Phage titer: 1.8 × 10^{-5} PFU/mL against the host Raoultella ornithinolytica (A1).

Transmission electron microscopy (TEM) analysis provided detailed structural measurements of the isolated bacteriophage (Figure 4). The virion exhibited a total length of 277 nm, featuring a distinct head-and-tail structure. The capsid (head) measured 91 nm in width and 116 nm in height, while the tail assembly showed a length of 119 nm and a width of 50 nm. At the distal end of the tail, a baseplate was identified with dimensions of 86 nm in width and 40 nm in height.

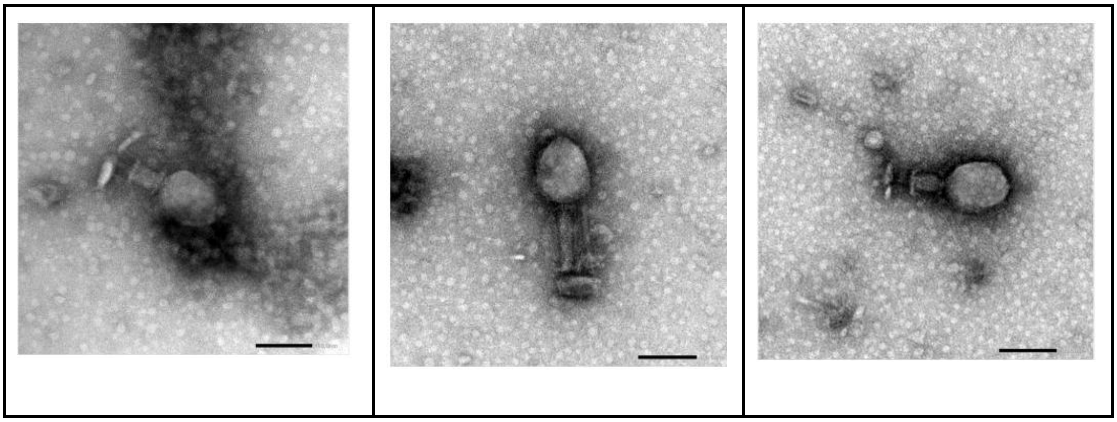

Figure 4 Transmission electron microscopy (TEM) micrograph of bacteriophage GA-A1-1. The image shows the structural morphology, including the icosahedral capsid, the tail assembly, and the baseplate structure. The scale bar corresponds to 100 nm.

The isolated bacteriophage GA-A1-1, previously enriched, was subjected to an assay to determine the optimal Multiplicity of Infection (MOI) against its host strain, *Raoultella ornithinolytica* (A1). Phage amplification was maximized at a low MOI, with the highest viral titer obtained at a MOI of 0.0007 (1.2×10^5 PFU/mL). Higher MOI values resulted in reduced or undetectable plaque formation (Figure 5). This observation is consistent with previous reports describing low optimal MOIs for efficient phage propagation (Wan et al., 2026).

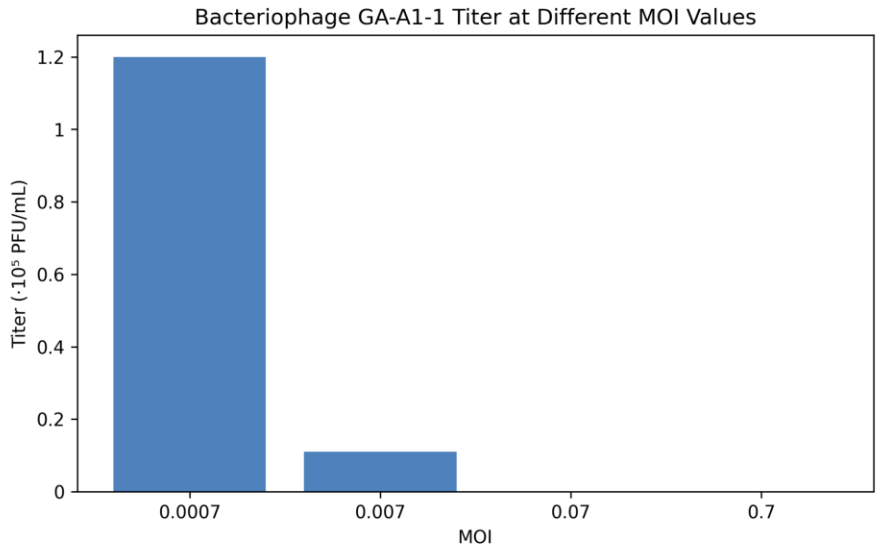

Figure 5 Phage titers of bacteriophage GA-A1-1 at different multiplicities of infection (MOI) against Raoultella ornithinolytica (A1). The lowest MOI (0.0007) yielded the highest titer (1.2 × 10^5 PFU/mL), whereas higher MOIs reduced or prevented plaque formation

DISCUSSION:

The presence of *R. ornithinolytica*, *C. freundii*, and *P. mirabilis* in wastewater samples underscores the technical challenge of managing antibiotic resistance within urban water cycles. Consistent with the findings of Mutuku, Gazdag, and Melegh (2022), our results suggest that treated effluent may serve as a vehicle for antibiotic resistance genes, facilitating their transfer between commensal and pathogenic bacterial populations in the environment. The detection of plasmid-bearing isolates in this study confirms that these systems are key points for understanding the flow of genetic resistance determinants, emphasizing the importance of ongoing research into advanced AMR control approaches.

The successful isolation of the bacteriophage GA-A1-1 from a WWTP, using the MDR *Raoultella ornithinolytica* (A1) as a host, confirms that wastewater environments are not only reservoirs of resistant bacteria but also rich sources of their natural predators. These findings align with the objective of the FAGOAQUA project, identifying wastewater-derived phages as promising biological agents for the control of environmental MDR bacteria that persist through conventional treatment processes.

TEM analysis revealed that GA-A1-1 possesses an icosahedral capsid and a distinct tail, consistent with bacteriophages belonging to the *Caudoviricetes* class within the *Duplodnaviria* domain. According to the morphological criteria of Giri (2021), the presence of a baseplate and tail assembly suggests an affiliation with tailed phages traditionally assigned to the *Myoviridae* or *Siphoviridae* families. Nevertheless, it should be noted these morphology-based families have been recently abolished by the ICTV (International Committee on Taxonomy of Viruses) in favor of a genome-based taxonomic framework (Valencia-Toxqui & Ramsey, 2024).

The ability of the viral isolate to effectively lyse *Raoultella ornithinolytica* at an optimal MOI of 0.0007 confirms the recovery of a robust lytic agent suitable for environmental biocontrol. Previous studies, such as that of Mirza et al. (2025), have demonstrated that bacteriophages isolated from sewage environments can maintain stable lytic activity against MDR clinical isolates, highlighting their potential as complementary strategies where conventional antibiotics are ineffective.

In this context, the characterization of phage GA-A1-1 aligns with current scientific trends that position bacteriophages as essential biotechnological tools for optimizing wastewater treatment. In conclusion, the isolation of GA-A1-1 represents a significant milestone within the FAGOAQUA project, proving that phage therapy is a viable, specific, and sustainable alternative to address the challenge of MDR bacteria in the urban water cycle.

METHODS:

Sampling and Isolation of Antimicrobial-Resistant Bacterial Strains

Wastewater samples were collected from two Basque WWTPs. To isolate resistant strains, an enrichment culture technique was employed using Luria-Bertani (LB) broth supplemented with specific selective antibiotics: ampicillin (100 µg/ml) and nalidixic acid (30 µg/ml). After an incubation period of 24 hours at 37°C, samples were streaked onto solid agar plates to obtain pure cultures of bacteria.

Microbiological Characterization and Pathogenicity Assessment

The isolated resistant strains underwent morphological and biochemical characterization. Cellular morphology was assessed using Gram staining. The pathogenic potential of the isolates was estimated using the Congo Red (CR) agar method (Berkhoff & Vinal, 1986; Cho et al., 2022).

Genetic Characterization via 16S rRNA Analysis

To achieve definitive taxonomic identification of the resistant isolates, genetic characterization was performed through 16S rRNA gene sequencing. Genomic DNA was extracted from pure cultures using the Nucleospin® Tissue kit (Macherey-Nagel, Düren, Germany). Extracted DNA was visualized using horizontal agarose electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA). The V3-V4 hypervariable regions of the 16S rRNA gene were amplified and subsequently sequenced. These assays were outsourced to the Advanced Research Facilities (SGIker) of the University of the Basque Country (UPV/EHU) and conducted according to the method described by Sanger et al. (1977), utilizing fluorochrome labeling and subsequent capillary electrophoresis. The resulting sequences were processed and compared against the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) to determine the phylogenetic identity of the strains (Altschul et al., 1990; Camacho et al., 2009).

Antimicrobial Sensitivity Testing

The antimicrobial susceptibility profiles of the isolated strains were determined using the disk diffusion method (Kirby-Bauer) on Mueller-Hinton agar (Merck KGaA, Darmstadt, Germany). The selection of antibiotic discs was based on their clinical relevance and included representative agents from various classes: GMN 10 (aminoglycosides), AMC 20/10 (penicillins combined with a beta-lactamase inhibitor), CIP 5 (fluoroquinolones), CZN 30 (third-generation cephalosporins), TET 30 (tetracyclines), VAN 30 (glycopeptides), ERY 15 (macrolides), CMN 10 (lincosamides), and CEF 30 (first-generation cephalosporins). The methodology and the breakpoints for determining resistance and susceptibility were established according to the standards and guidelines provided

by the Spanish Society of Infectious Diseases and Clinical Microbiology (García Rodriguez et al., 2000).

Isolation and Enrichment of Lytic Bacteriophages

Wastewater samples were collected from Basque WWTPs. Sample clarification and lytic bacteriophage isolation were carried out following the methodology described by Runa et al. (2021). Prior to isolation, samples were clarified by low-speed centrifugation to remove suspended solids and subsequently filtered to eliminate bacterial debris. Lytic bacteriophages were isolated using an enrichment procedure with appropriate bacterial host strains, followed by plaque assay techniques to detect phage activity. Individual plaques were selected and subjected to successive rounds of purification to ensure clonal phage populations before further characterization.

***In Vitro* Determination of Bacteriolytic Effect**

The bacteriolytic activity of the isolated phages was evaluated through liquid culture assays as described by Liu et al. (2020). The Multiplicity of Infection (MOI) was calculated as the ratio of infectious viral particles (PFU) to host bacterial cells (CFU). Standardized bacterial suspensions in LB broth were challenged with specific phage titers to achieve different MOI levels, ensuring bacterial infection at high ratios.

Morphological Characterization via TEM

The morphological analysis of the isolated bacteriophages was performed to determine their structural characteristics and taxonomic classification. This assay was outsourced to the Advanced Research Facilities (SGIker) of the University of the Basque Country (UPV/EHU). Phage samples were prepared following a protocol adapted from Pallavali et al. (2023). For contrast enhancement, the samples were negatively stained with a uranyl acetate solution. The grids were examined using a JEM-1400-Plus transmission electron microscope (JEOL Ltd., Tokyo, Japan). Morphological measurements and image analysis were conducted using the ImageJ 1.53m online software (Ferreira & Rasband, 2011).

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FIGURES:

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DATA AVAILABILITY STATEMENT:

The datasets generated and analysed during the current study (including sequencing data, Gram staining results, and antimicrobial susceptibility profiles) are available from the corresponding author on reasonable request.

COMPETING INTERESTS STATEMENTS:

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.