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Research article

First identification of multidrug-resistant *Acinetobacter bereziniae* isolates harboring *bla*$_{\text{NDM-1}}$ from hospitals in South China

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ABSTRACT

This study is a first report on the identification of multidrug-resistant (MDR) *Acinetobacter bereziniae* among non-*baumannii* acinetobacters that had previously escaped automated laboratory detection, and characterize their clinical courses of infection at two tertiary-care hospitals in Shenzhen city, China (2015–2017). Herein, definitive identification by PCR was performed with universal and species-specific primers targeting 16S rDNA and *rpoB* genes, respectively, followed by Sanger sequencing and blast analysis. Antimicrobial susceptibility of *A. bereziniae* isolates was assessed accordingly. Three of the five identified *A. bereziniae* isolates exhibited carbapenem-resistance and were subjected to a multiplex PCR assay to detect drug-resistance genes. Sequences of the *rpoB* amplicon were aligned with curated sequences from global databases for phylogenetic analysis on evolutionary relations. Five clinical isolates of *A. bereziniae* were thereby re-identified, whose infections were primarily nosocomial. Automated identification and susceptibility testing systems (Phoenix-100 and VITEK 2) proved insufficient for discriminating *A. bereziniae* from other acinetobacters such as *Acinetobacter baumannii* and *Acinetobacter guillouiae*. Among these isolates, three exhibited carbapenem-resistant phenotypes indistinguishable from that of carbapenem-resistant *A. baumannii*. The carbapenem-resistant *A. bereziniae* isolates were subsequently confirmed to carry a *bla*$_{\text{NDM-1}}$ (New Delhi metallo-β-lactamase-1) gene downstream of IS*Aba125*. Phylogenetic analysis revealed that *A. bereziniae* isolates evolved slowly but independently in local habitats. *A. bereziniae* isolates are difficult to distinguish by traditional

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automated detection systems. PCR-based identification via amplification and sequencing of selected house-keeping genes provides sufficient resolution for discriminating the isolates.

1. Introduction

A critical lack of incentives for developing new antibiotics and uncritical use of antimicrobials in hospital and farming settings have hastened the advent of the post-antibiotic era [1]. This problem is even more pronounced in regions where traditions of epidemiological surveys and antimicrobial stewardship have been weak. Extensive use of a finite set of broad-spectrum antibiotics spurs the spread of multidrug/extensively drug-resistant (MDR/XDR) nosocomial pathogens, particularly Gram-negative bacteria. Notably, *Acinetobacter* spp. are a group of opportunistic pathogens that have posed increasing health risks in the hospital environment, as their rates of associated infections, resistance, and morbidity/mortality continue to increase across the world [2]. Although *Acinetobacter baumannii* has conventionally been deemed the most prominent pathogenic *Acinetobacter* species in healthcare-associated infections (HAIs) [3, 4], isolates of other members of the *Acinetobacter* genus including *A. nosocomialis*, *A. pittii*, *A. seifertii, A. soli*, and *A. ursingii*, are also frequently detected [5, 6, 7], some of which possess pathogenic potential as does *A. baumannii*.

Conventionally, automated identification and susceptibility testing systems including Phoenix-100 (BD Diagnostics, MD, USA) and VITEK 2 (bioMérieux, Marcy l'Etoile, France) are the mainstay approach to rapid detection of pathogenic bacteria in hospitals. However, these systems are built on databases that sometimes lack information on emerging pathogens. This occasionally gives rise to uncertainties such as mis-identification and underdiagnosis in the absence of confirmative evidence by molecular approaches including PCR and WGS (whole-genome sequencing). A major unaddressed issue in preventing and treating infections by non-*baumannii Acinetobacter* species has been an unmet need for methods allowing accurate and rapid identification of potential pathogens. Distinguishing non-*baumannii Acinetobacter* species from *A. baumannii* is essential for ensuring accurate diagnosis and optimized treatment [8].

In this study, we report an underappreciated incidence of detection of drug-resistant *Acinetobacter bereziniae* (previously *Acinetobacter* genomospecies 10) isolates from two large tertiary care hospitals in Guangdong province, South China. During our recent epidemiological studies on 324 clinical *A. baumannii* isolates from the hospitals, approximately 10% (28 isolates) of all these isolates were found to be initially mis-identified by automated detection systems (Phoenix-100 and VITEK 2). Among these, a total of five *A. bereziniae* isolates were identified by 16S rDNA and *rpoB* sequences, as well as a species-specific PCR. Three isolates displayed a carbapenem-resistant phenotype indistinguishable from that of carbapenem-resistant *A. baumannii* (CRAB). Surprisingly, the carbapenem-resistant isolates proved to be carriers of a $bla_{\text{NDM-1}}$ (New Delhi metallo-β-lactamase-1) gene downstream of IS*Aba125*, which arms them as problematic isolates.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Five clinical isolates of *A. bereziniae* investigated in this study were collected during the period of 2015–2017 by medical laboratories of two major tertiary care hospitals (Shenzhen People's Hospital and Shenzhen Third People's Hospital) in Shenzhen city, Guangdong province, China. Two *A. baumannii* type strains ATCC 19606 and ATCC 17978, as well as seven other clinical *Acinetobacter* spp. isolates GD0302, GD0307, GD0308, GD0318, GD03184, GD03186 and GD03210, served as references. Isolates and strains used in this study were cultured in Luria-Bertani broth shaking at 37 °C. Pharmacological experiments were performed in Mueller-Hinton liquid culture or agar plates.

Identification of A. bereziniae and detection of $bla_{\text{NDM-1}}$ *in A. bereziniae by PCR.*

5wInitially, the identity of clinical isolates was determined as *Acinetobacter* spp. by phenotypic identification methods such as the Phoenix-100 system (BD Diagnostics) or VITEK 2 system (bioMérieux). In order to distinguish *Acinetobacter* spp. from other species, universal 16S rDNA primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [9] were used. To further discriminate *A. bereziniae* from other *Acinetobacter* spp., PCR targeting *rpoB* (RNA polymerase subunit β) was performed in two different manners: Firstly, with Ac-rpoBz2-1055F (5′-GTGATAARATGGCBGGTCGT-3′) and Ac-rpoBz2-1598R (5′-CGBGCRT GCATYTTGTCRT-3′) [10] to generate a 511-bp amplicon for sequencing of *rpoB* (zone 2); and secondly, with the species-specific primers Aber-F (5′-ACGGTTCTCCGGGCGTCT-3′) and Aber-R (5′-AACACCACCTTCAGCGATTTTA-3′) [11] to give an expected 539-bp amplicon unique to *A. bereziniae*. Similarly, to detect $bla_{\text{NDM-1}}$, PCR was performed with a primer mix containing ISAba125-F (5′-ACTGTCGC ACCTCATGTTTG-3′), NDM1-F (5′-GTCTGGCAGCACACTTCCTA-3′) and NDM1-R (5′-TAGTGCTCAGTGTCGGCATC-3′) [12] to generate a 515-bp amplicon in the coding sequence of $bla_{\text{NDM-1}}$, as well as a larger PCR product to determine the association of IS*Aba125* and $bla_{\text{NDM-1}}$. PCR products were subjected to Sanger sequencing, where appropriate.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility profiles of the clinical isolates were primarily determined by the Phoenix-100 system (BD Diagnostics), whose results were interpreted according to MIC interpretive standards of the CLSI (2019) (Clinical and Laboratory Standards Institute, 2019) [13] for *Acinetobacter* spp. Additional confirmative tests for colistin, gentamicin and amikacin were performed by the broth microdilution method.

2.3. Phylogenetic analysis of A. bereziniae isolates

For phylogeny, to obtain corresponding DNA sequences of *rpoB* (zone2) in clinical *A. bereziniae* isolates, PCR was performed as described above with the primer pair Ac-rpoBz2-1055F (5′-GTGATAARATGGCBGGTCGT-3′) and Ac-rpoBz2-1598R (5′-CGBGCRTGCATYTTGTCRT-3′) [10] and the resultant PCR products were subsequently subjected to Sanger sequencing. The sequences of *rpoB* of five *A. bereziniae* isolates in this study were grouped with 22 additional *A. bereziniae* (*Acinetobacter* genomic species 10), 4 *A. guillouiae* (*Acinetobacter* genomic species 11) and 2 representative *A. baumannii* isolates deposited in the NCBI database for phylogenetic analysis performed by MEGA7 using the Neighbor-Joining method.

3. Results

3.1. Patient cases and clinical courses of A. bereziniae infection

As five *A. bereziniae* isolates were serendipitously collected in this study, the patient cases with associated infections were examined to better understand their health consequences. As summarized in Table S1, all five *A. bereziniae* isolates were collected from the two hospitals during 2015–2017. Specifically, two isolates, GD03392 and GD03393, were recovered at SZPH, from eye mucus of a neonate, and blood of an adult patient after neurosurgical operation, respectively. The remaining three isolates, GD0320, GD03185 and GD03255, were obtained at SZTPH from sputum.

To characterize the course of infection by *A. bereziniae*, medical records of corresponding patients were extracted and analyze. In addition, we consulted previous reports on *A. bereziniae* infections and summarized their findings as listed in Table 1 for comparison. In total, 16 documented cases in the literature and 5 currently reported cases have been registered up to late 2020. Typically, patients were of older age or had suffered severe underlying disease(s) on admission. Acquisition route of *A. bereziniae* was frequently classified as nosocomial, which suggests that risk factors similar to that of *A. baumannii* infection may be important. After combinatorial treatments of antibiotics, most of the infected patients were cured, with only one death case being reported (Figure 1). This suggests that *A. bereziniae* remains generally a minor opportunistic pathogen in the hospital environment, and its potential to cause fatal infections was relatively low. However, in our study, patients admitted to departments other than ICU (GD03255, GD03393) and even an 11-day neonate (GD03392) were infected by *A. bereziniae*. Given that many hospitals may not be technically able to perform PCR- or DNA sequencing-based identification of the pathogen, we reason that the prevalence and pathogenic potential of *A. bereziniae* may well be underestimated.

Re-identification of A. bereziniae among mis-identified non-baumannii acinetobacters.

During our recent efforts to conduct epidemiological surveys on 324 clinical *A. baumannii* isolates from eight major hospitals in Guangdong province, China, we noticed that approximately 10% of all clinical *Acinetobacter* isolates (28/324) identified by automated detection systems might not be *A. baumannii*. Since a small but unusual fraction of these isolates exhibited extensive drug resistance, we were motivated to clarify their true identities and potential health risks to patients. By adopting a double-test strategy via PCR-based identification of 16S rDNA and *rpoB* sequences, we successfully ascertained the identities of all these clinical isolates other than *A. baumannii*. Most of them (19/28) were confirmed to be various *Acinetobacter* spp. (Table S2), and the rest were *Enterobacteriaceae*, *Xanthomonadaceae*, and *Micrococcaceae* (Data not shown). Further analysis of such *Acinetobacter* spp. indicated that the proportion of a particular species of *A. bereziniae* from two major hospitals (SZPH and SZTPH) was unexpectedly high (5/19) (Table S2), accompanied by AST profiles of relatively high drug resistance. This prompted us to examine their molecular determinants.

The clinical *A. bereziniae* isolates GD0320, GD03185 and GD03255 from SZTPH, and GD03392, GD03393 from SZPH, were initially identified as *A. baumannii* at the medical laboratories (Table S1). However, MLST (multi-locus sequence typing) analysis of such isolates consistently proved difficult or failed, suggesting they might be species other than *A. baumannii*. To clarify their identities, we first amplified two conserved regions on 16S rDNA and *rpoB* (zone2), and the PCR products were subsequently subjected to sequencing. Sequence alignment results suggest that the isolates were in fact *A. bereziniae*, having high similarities with *A. baumannii* (96–97%) in 16S rDNA (Figure S1) and apathogenic *A. guillouiae* (~95%) in *rpoB* (zone 2) (Figure S2). For confirmation of the species' identity, we used species-specific primers [11] to perform PCR with genomic DNA from a number of *Acinetobacter* spp. PCR amplification yielded products only for *A. bereziniae* isolates, while neither *A. baumannii* nor other *Acinetobacter* spp. generated any amplicon (Figure 2a).

3.2. Antimicrobial susceptibility profiles of A. bereziniae isolates

Based on AST evaluation (Phoenix-100 system, BD) and interpretation according to MIC interpretive standards of the CLSI (2019) for *Acinetobacter* spp., two isolates (GD0320 and GD03392) showing resistance to few (0–2) drugs were assigned as susceptible isolates (S-type). Three other isolates (GD03185, GD03255 and GD03393) exhibiting multidrug resistant profiles (with resistance against >8 drugs) were determined as resistant isolates (R-type) (Table 2). Remarkably, the three R-type isolates showed resistance to all tested β-lactam classes of antibiotics including cephalosporins and carbapenems (MIC of imipenem ≥8 μg/mL and meropenem >8 μg/mL), while all five isolates remained susceptible to amikacin (MIC ≤8 μg/mL) and colistin (i.e. polymyxin E; MIC ≤1 μg/mL), with variable susceptibilities to gentamicin and trimethoprim-sulfamethoxazole.

3.3. Detection of bla_{NDM-1} and other antimicrobial resistance genes

The AST results suggest that three R-type *A. bereziniae* isolates (GD03185, GD03255 and GD03393) displaying carbapenem-

Table 1
Characteristics of twenty-one patients with *A. bereziniae* infections reported in literature and this study.

Isolate	Age/ Sex[#]	Status at admission[#]	Underlying disease[#]	Acquisition[#]	Treatment[#]	NDM-1 carriage[#]	Outcome[#]	Reference
KH243	NR	NR	NR	NR	NR	NR	NR	[14]
CHI-40-1	NR	NR	NR	NR	NR	Yes	NR	[16]
HPC229	53 y/F	Severe sepsis	Leukemia	Nosocomial	Ciprofloxacin, tigecycline	Yes	Died	[17]
KR107539	21 y/M	NR	NR	Nosocomial	NR	Yes	NR	[18]
Nec	NR	NR	NR	Nosocomial	NR	NR	NR	[20]
Baz	NR	NR	NR	Nosocomial	NR	NR	NR	[20]
NR	NR	NR	Chronic obstructive pulmonary disease	Nosocomial	NR	NR	NR	[21]
HGSA593	NR	NR	Chronic renal disease	Nosocomial	NR	NR	NR	[22]
YMC 05/10/ R155	40 y/M	Colonised, high fever	Acute myelogenous leukaemia	Nosocomial	Ciprofloxacin, metronidazole, quinupristin/dalfopristin	NR	Survived	[23]
YMC 06/10/ R1547	54 y/M	Colonised, high fever	Status epilepticus, acute renal failure, diabetes insipidus	Nosocomial	Ceftriaxone, amikacin, clindamycin, vancomycin	NR	Died	[23]
YMC 06/11/ U1908	64 y/M	Urinary tract infection	Corona radiata infarction, chronic renal failure, hypertension	Nosocomial	Ceftriaxone, clindamycin, minocycline	NR	Survived	[23]
YMC 07/1/ R556	66 y/M	Pneumonia, sepsis	Myocardial infarction, cerebral edema	Nosocomial	Ceftriaxone, clindamycin, panipenem, vancomycin, amikacin	NR	Died	[23]
BDC 07/PDR	63 y/F	Urinary tract infection	Meningioma	Nosocomial	Cefodizime, cefixime, isepamicin, ciprofloxacin, levofloxacin	NR	Survived	[23]
P154	NR	NR	NR	NR	NR	Yes	NR	[19]
L65	62 y/M	Traumatic injuries	NR	Nosocomial	Meropenem, ampicillin/sulbactam	No	Survived	[24]
L98	66 y/F	Cough, dyspnea	Bronchial infection, congestive heart failure	Nosocomial	Levofloxacin, vancomycin + piperacillin/tazobactam	No	Died	[24]
GD0320	40 y/M	Head injury, coma	Severe craniocerebral injury	Nosocomial	Cefoperazone/tazobactam, cefuroxime, ceftazidime, cefazolin, piperacillin/tazobactam, imipenem	No	Died	This study
GD03185	59 y/F	Dysphagia, muscle pain	Tetanus infection	Nosocomial	Penicillin, metronidazole	Yes	Survived	This study
GD03255	35 y/F	NA	ND	ND	ND	Yes	Survived	This study
GD03392	11 d/ M	Yellowing skin	Neonatal pathologic jaundice, conjunctivitis	Nosocomial	Tobramycin	No	Survived	This study
GD03393	64 y/M	Spontaneous hemorrhage, coma	Multiple organ dysfunction syndrome	Nosocomial	Biapenem, vancomycin, linezolid, aztreonam, amikacin, cefoperazone/sulbactam, imipenem	Yes	Discharged	This study

[#] NR: not reported; ND: not determined; NA: not applicable.

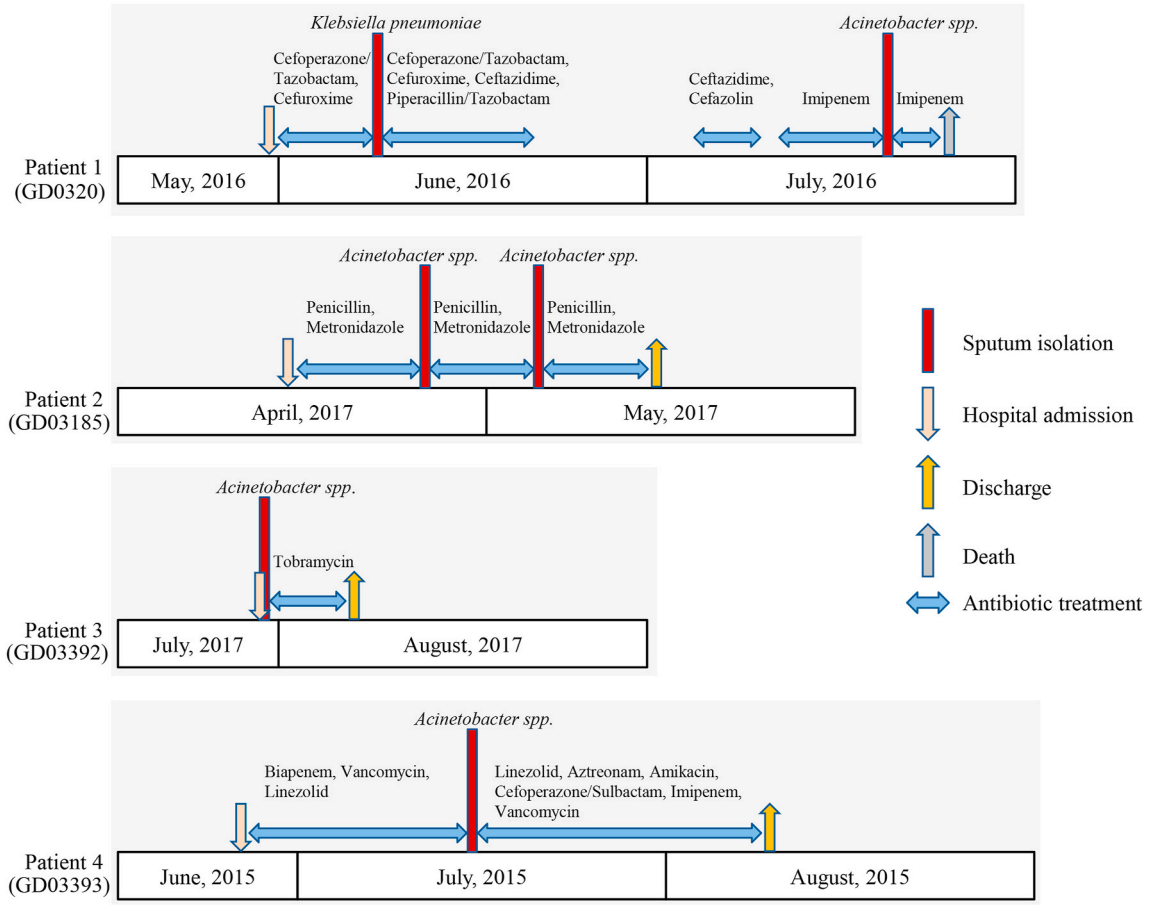

Figure 1. Course of disease among inpatients during infection by *A. bereziniae*. The clinical course of disease during infection by an *A. bereziniae* isolate (GD0320, GD03185, GD03392 and GD03393), including admission, sampling, identification, use of antibiotics and clinical outcomes, is schematically summarized. The *A. bereziniae* isolate GD03255 was collected from an outpatient, who had no corresponding records. Upon treatments including antibiotics, three patients were cured, and one died eventually.

resistant phenotypes might harbor genetic elements that enable resistance against carbapenems. OXA-like carbapenemase genes (e.g., $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, or $bla_{\text{OXA-58-like}}$), the acquired β-lactamases most commonly detected in *Acinetobacter* spp., were initially selected as candidate targets for detection in multiplex PCR. However, only the isolates GD0320 and GD03393 gave positive results for $bla_{\text{OXA-58-like}}$, and the remaining OXA-like carbapenemase genes were tested negative (Data not shown). To reconcile these seeming discrepancies between observed carbapenem resistance and non-detection of OXA-like carbapenemase genes, we set out to attempt PCR-based detection of other key carbapenemase gene(s), especially $bla_{\text{NDM-1}}$. When a multiplex PCR with IS*Aba125*-F/NDM1-F/R primers was conducted, total three bands were obtained for all three R-type isolates, which included a 515-bp PCR product for $bla_{\text{NDM-1}}$, and two larger bands (Figure 2b). Through further analyses by PCR, sequencing and alignments, we have gained a better picture of determinants for carbapenemase activity in carbapenem-resistant *A bereziniae*. Firstly, the fragment slightly larger than 750 bp represents a combination of partial IS*Aba125* and $bla_{\text{NDM-1}}$, implying that $bla_{\text{NDM-1}}$ in the three R-type isolates is located downstream of IS*Aba125* (Figure S3). Secondly, the sequences of this amplified fragment were identical (Figure S3). Thirdly, the largest band of approximately 1,500 bp seemed to be a fragment flanked by two copies of IS*Aba125*, since the corresponding amplicon could also be obtained with a solo ISAba125-F primer using genomic DNA from such R-type isolates (Data not shown). This ~1,500-bp PCR product could not be sequenced with the IS*Aba125*-F primer, but it does suggest that all the R-type *A. bereziniae* isolates had been subjected to repeated transpositional events that gave rise to multiple copies of IS*Aba125*, possibly on the bacterial chromosome. Nevertheless, the genomic features and genetic variations such as the precise location of IS*Aba125*-$bla_{\text{NDM-1}}$ in the R-type isolates remain obscure, which forms the subject matter of our on-going studies.

3.4. Evolutionary relations of A. bereziniae among clinically relevant acinetobacter species

As noted earlier, clinical infections by *A. bereziniae* share characteristics with that of *A. baumannii*. We next ventured to analyze the phylogeny of *A. bereziniae* isolates to map out their evolutionary relationships and distances relative to other acinetobacters. A

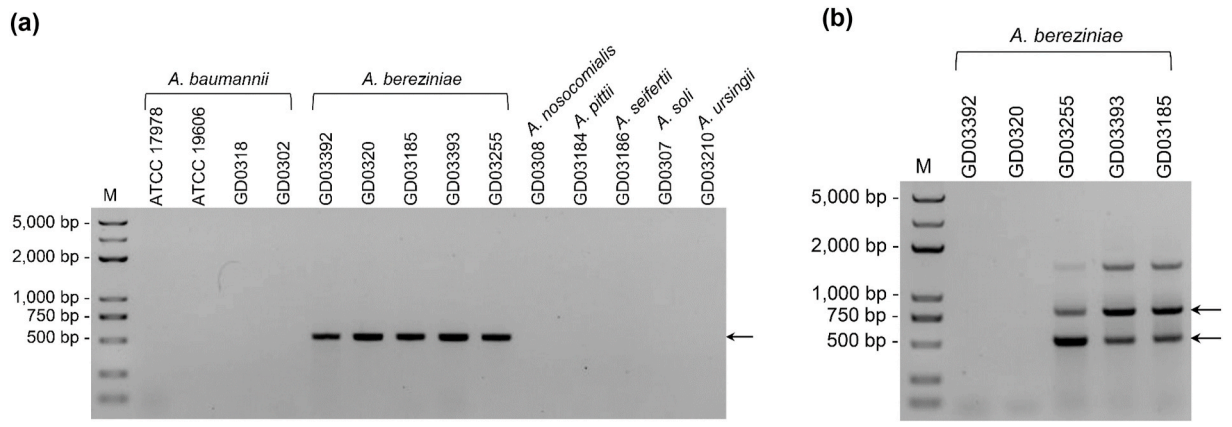

Figure 2. Identification of *A. bereziniae* and validation of bla_{NDM-1} by PCR. **(a)** Gel electrophoresis (SI2) for *A. bereziniae* identification by using species-specific primers in 14 *Acinetobacter* spp. isolates. **(b)** Gel electrophoresis (SI3) for bla_{NDM-1} validation within 5 clinical *A. bereziniae* isolates. Expected PCR products are indicated by an arrow(s) next to the image.

Table 2
Antimicrobial susceptibilities of *A. bereziniae* clinical isolates.

Isolate	MIC (μg/mL)[#]											
	SAM	TZP	CAZ	CTX	FEP	IPM	MEM	CIP	AMK	GEN	SXT	COL
GD03392	≤4 (S)	≤4 (S)	8 (S)	8 (S)	4 (S)	≤1 (S)	≤1 (S)	≤0.5 (S)	≤8 (S)	≤2 (S)	≤0.5 (S)	≤0.5
GD0320	≤4 (S)	16 (S)	>16 (I)	32 (I)	4 (S)	≤1 (S)	≤1 (S)	>2 (R)	≤8 (S)	≤2 (S)	>2 (R)	1
GD03185	>16 (R)	64 (I)	>16 (R)	>32 (R)	>16 (R)	8 (R)	>8 (R)	>2 (R)	≤8 (S)	4 (S)	>2 (R)	1
GD03393	>16 (R)	>64 (R)	>16 (R)	>32 (R)	>16 (R)	>8 (R)	>8 (R)	>2 (R)	≤8 (S)	>8 (R)	>2 (R)	1
GD03255	>16 (R)	>64 (R)	>16 (R)	>32 (R)	>16 (R)	>8 (R)	>8 (R)	>2 (R)	≤8 (S)	8 (I)	>2 (R)	1

[#] MIC was determined by the BD Phoenix-100 system. Antimicrobial susceptibility results in parentheses were based on MIC interpretive standards of the CLSI (2019) for *Acinetobacter* spp.: R, resistant; I, intermediate; S, susceptible. SAM: ampicillin-sulbactam; TZP: piperacillin-tazobactam; CAZ: ceftazidime; CTX: cefotaxime; FEP: cefepime; IPM, imipenem; MEM, meropenem; CIP: ciprofloxacin; AMK: amikacin; GEN: gentamicin; SXT: trimethoprim-sulfamethoxazole; COL: colistin (polymyxin E).

phylogenetic tree, comprising 27 global isolates of *A. bereziniae*, 4 *A. guillouiae* isolates, and 2 representative *A. baumannii* isolates, was constructed based on our own and previously published *rpoB* (zone 2) sequences. The tree was appended with information on years of report and countries of origin (Figure 3). Although *A. guillouiae* has been proposed to be an *Acinetobacter* species most genetically akin to *A. bereziniae*, they are actually separated in two minor clades and grouped together into a major clade, displaying a considerable evolutionary distance from the clade to which *A. baumannii* belongs. Within the clade of *A. bereziniae*, remarkable variability was observed. Neither *A. bereziniae* isolates collected from different continents (Asia, Europe and South America) across the years, nor isolates of bla_{NDM-1} carriage in the present study are neatly grouped into a minor clade, but they are instead unevenly distributed and mixed together. This indicates that *A. bereziniae* as a pathogen may have undergone slow yet independent evolutions in different local habitats.

4. Discussion

In this study, *Acinetobacter* isolates initially mis-identified by two well-known automated ID/AST testing systems (Phoenix-100 and VITEK 2) were investigated. It is possible that misidentification of genetically related species could give rise to erroneous diagnosis and misguide treatment against emergent pathogens. Understandably, limitations inherent to the sensitivity and resolution of the automatic detection systems will take some time to be overcome. For poorly characterized pathogens such as *A. bereziniae* [14], it seems essential to develop reliable and cost-effective approaches to quickly pin down infectious causes in definitive diagnostics, e.g., molecular assays on genomic markers of species and antimicrobial resistance genes, so as to safeguard rational use of antibiotics and improve treatment outcomes. Discovery of emerging pathogens, especially those with extensive drug resistance potential, is also an urgent priority contingent on our ability to detect and differentiate underdiagnosed pathogens by informed approaches.

Here, we reported five clinical *A. bereziniae* isolates collected from two tertiary care hospitals in South China over a period of three years (2015–1017). These initially mis-identified isolates positively identified as *A. bereziniae* by PCR coupled with Sanger sequencing for 16S rDNA and *rpoB*. Sequence alignments of the two genes (Figure S1 and S2) indicate that both the S-type (GD0320 and GD03392) and R-type isolates (GD03185, GD03255 and GD03393) had little difference in conserved genes or core genes. For the three NDM-positive isolates, they were collected from different ward/hospital sources over several years (Table 1 and 3) and found to have

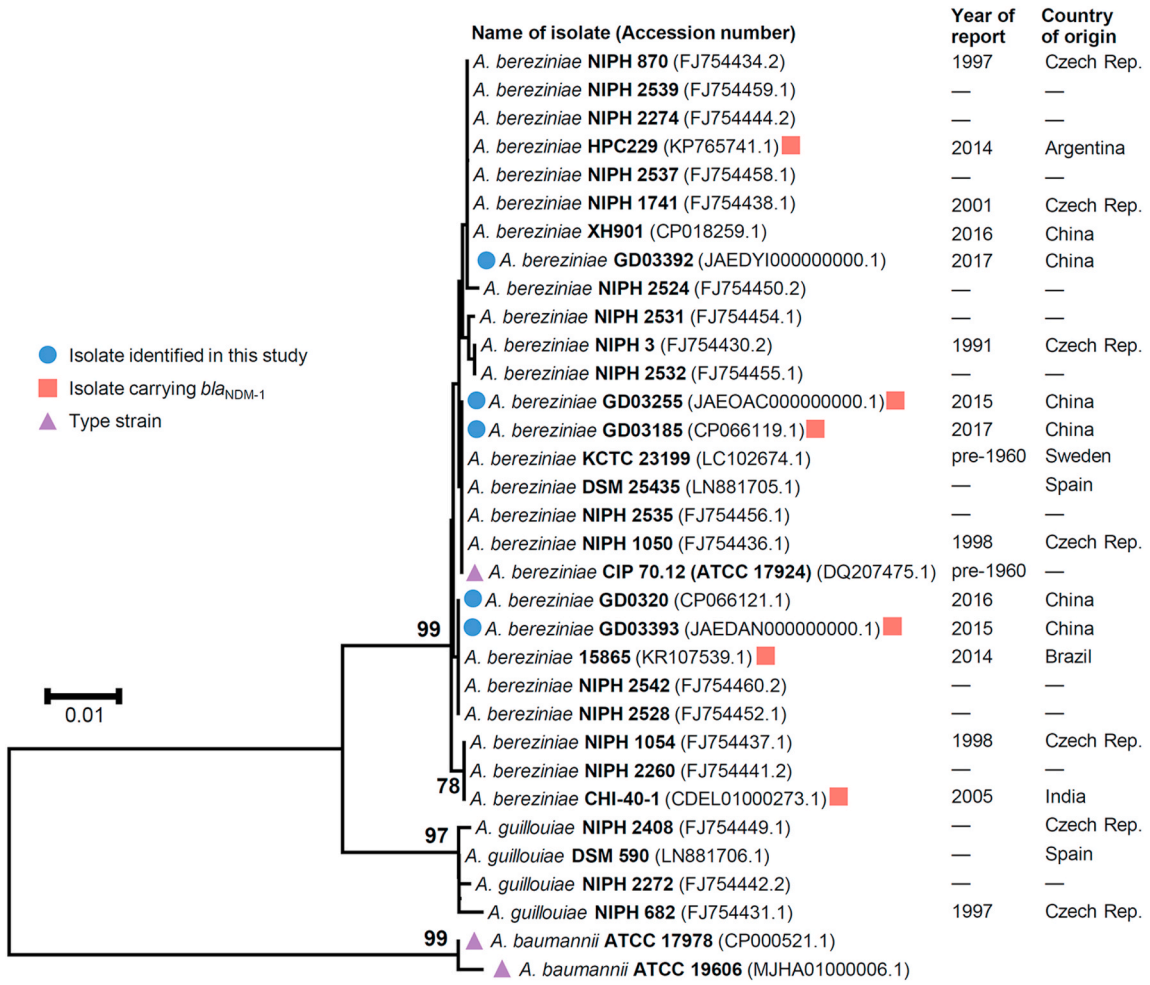

Figure 3. Phylogenetic tree of all 27 published global *A. bereziniae* isolates, 4 *A. guillouiae* isolates, and 2 representative *A. baumannii* type strains based on *rpoB* (zone 2) sequence. Five *A. bereziniae* isolates identified in this study are labelled with a filled circle (●); type strains of *A. bereziniae* and *A. baumannii* are labelled with an open circle (○); isolates carrying bla_{NDM-1} are labelled with a filled square (■). GenBank accession numbers for *rpoB* are shown in parentheses. Years of report and countries of origin for the isolates are indicated next to a strain. Cluster analysis (Neighbor-Joining) was conducted with MEGA7. Percentages of replicate trees (>70%) in which the associated taxa clustered together in bootstrap tests (1,000 replicates) are shown above the branches. The tree is drawn to scale, with branch lengths in the same units as those of evolutionary distances.

non-identical *rpoB* (zone 2) sequences. This seems to favor the conjecture that such carbapenem-resistant isolates have independently undergone genetic evolution to acquire bla_{NDM-1}.

In the search for molecular determinants of carbapenem resistance in the three R-type isolates, we identified all three to be carriers of bla_{NDM-1}, which is unexpected given the relatively small sample pool. This situation is nonetheless alarming as the spread of bla_{NDM-1} may be ongoing among local *Acinetobacter* species. Being an opportunistic pathogen, *A. bereziniae* could potentially serve as a reservoir facilitating the dissemination of bla_{NDM-1} to other nosocomial bacteria. To prevent this, care should be taken to intensify monitoring of such emerging pathogens and antimicrobial resistance genes in hospitals.

Intriguingly, through a preliminary survey on *A. bereziniae* isolates deposited within the *A. baumannii* library in the PubMLST database (pubmlst.org), 18 entries of worldwide *A. bereziniae* isolates were found listed (Figure S4). Except for CIP 70.12, seventeen *A. bereziniae* isolates were all reported after 2009, and distributed in at least nine countries across five inhabitable continents. These include 4 *A. bereziniae* isolates from China and two being reported (GD03185 and GD0320) in this study. Although the Oxford and Pasteur schemes of MLST were originally designed for molecular typing of *A. baumannii*, 17 of the 18 *A. bereziniae* isolates could be classified by using the Pasteur scheme. Under the Pasteur scheme, 14 different STs were found with no apparent dominant types, whilst classification by the Oxford scheme proved unsuccessful. The growing discoveries of *A. bereziniae* isolates from around the world suggests that *A. bereziniae* may have been living in the human host as an under-detected microbe for a long time, while its potential pathological implications remain unclear. Our work here verified 3 more *A. bereziniae* isolates carrying bla_{NDM-1} from HAIs, which again implicates it as a neglected species.

As a fast-growing metropolis bordering Hong Kong, Shenzhen has a population of over 13 million (2021). Massive and rapid flux of

migrant workers has likely added additional risks to the spread of bla_{NDM-1} between hospitals and communities. To this date, detection of bla_{NDM-1} has been occasionally reported in some *Acinetobacter* species, especially *A. baumannii*, worldwide [15]. However, only four similar cases were reported for *A. bereziniae*: from India (2005) [16], Argentina (2014) [17], Brazil (2014) [18] and Burkina Faso [19]. Ours is the first to report bla_{NDM-1} carriage in three distinct carbapenem-resistant clinical *A. bereziniae* isolates in China and the East Asian region. In previous studies from South Asia and South America, bla_{NDM-1} in *A. bereziniae* was reportedly located in Tn*125*, downstream of IS*Aba125* within a plasmid, which is presumably acquired via conjugation [16, 17, 18]. Our preliminary study also supported that bla_{NDM-1} might be on Tn*125*, but whether it is embedded in a plasmid remained uncertain, since several of our assays on plasmid(s) curing proved unsuccessful among the R-type strains (data not shown). As for species other than *A. bereziniae*, Tn*125* could occur either within plasmids by conjugation or chromosomes by transposition. Mechanistic details of antimicrobial resistance and virulence in *A. bereziniae* warrant further scrutiny, particularly in the context of whole-genome sequencing.

This work has been primarily motivated by the need for a better understanding of *A. bereziniae* as a locally emerging pathogen implicated in several cases of hospital-acquired infection in South China. Admittedly, however, it has some limitations. Namely, our findings focused mainly on molecular identification and detection of the bla_{NDM-1} gene; additional definitive diagnostic approaches and non-NDM-1 resistance genes were not discussed. The scope of the study was restricted in part by the small sample size available to us. Further investigation on the nature of resistance determinants (e.g. the intrabacterial location of bla_{NDM-1}) is currently underway with our genome sequencing data, whose results will be made available on completion of the analyses.

In conclusion, our work represents the first report on the detection of carbapenem-resistant clinical *A. bereziniae* isolates with bla_{NDM-1} carriage in China and in the East Asian region as a whole, which urges a greater awareness on drug-resistant *A. bereziniae* prevalence and spread in hospitals and the community. In view of the findings in this present study, we also think that it is recommendable to employ identification systems that are more robust in definitive identification of *Acinetobacter* species, such as MALDI-TOF and mNGS (metagenomic next-generation sequencing) or a combination of them to improve diagnostic resolution in clinical settings. Further studies on the phenotypic and genetic characteristics of *A. bereziniae* could provide insights into the pathogenicity and infection biology of this emerging pathogen.

Declarations

Author contribution statement

Xiao-Mei Mo: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Qing Pan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Harald Seifert, Paul G. Higgins: Analyzed and interpreted the data; Wrote the paper.

Xi-Wen Xing, Jing Yuan: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Zi-Yuan Zhou, Xing-Yu Luo, Hou-Ming Liu, Yong-Li Xie, Liu-Qing Yang, Xiao-Bing Hong: Performed the experiments.

Nai-Kei Wong: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Declaration of interest's statement.

The authors declare no competing interests.

Additional information

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