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Original Article

A 19-year longitudinal study to characterize carbapenem-nonsusceptible *Acinetobacter* isolated from patients with bloodstream infections and the contribution of conjugative plasmids to carbapenem resistance and virulence

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KEYWORDS
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Bacteremia;
bla$_{VIM}$;

Abstract *Background:* This study aimed to characterize carbapenem-nonsusceptible *Acinetobacter* (CNSA) isolated from patients with bacteremia from 1997 to 2015.
Methods: A total of 173 CNSA (12.3%) was recovered from 1403 *Acinetobacter* isolates. The presence of selected β-lactamase genes in CNSA was determined by PCR amplification. The

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bla$_{IMP}$;
Carbapenem resistance;
Carbapenemase;
Conjugative plasmid;
Virulence

conjugation test was used to determine the transferability of metallo-β-lactamase (MBL)-carrying plasmids. Whole genome sequencing in combination with phenotypic assays was carried out to characterize MBL-plasmids.

Results: In general, a trend of increasing numbers of CNSA was observed. Among the 173 CNSA, *A. baumannii* (54.9%) was the most common species, followed by *A. nosocomialis* (23.1%) and *A. soli* (12.1%). A total of 49 (28.3%) CNSA were extensively drug-resistant, and all were *A. baumannii*. The most common class D carbapenemase gene in 173 CNSA was *bla*$_{OXA-24-like}$ (32.4%), followed by IS*Aba1*-*bla*$_{OXA-51-like}$ (20.8%), IS*Aba1*-*bla*$_{OXA-23}$ (20.2%), and IS*1006*/IS*1008*-*bla*$_{OXA-58}$ (11.6%). MBL genes, *bla*$_{VIM-11}$, *bla*$_{IMP-1}$, and *bla*$_{IMP-19}$ were detected in 9 (5.2%), 20 (11.6%), and 1 (0.6%) CNSA isolates, respectively. Transfer of MBL genes to AB218 and AN254 recipient cells was successful for 7 and 6 of the 30 MBL-plasmids, respectively. The seven AB218-derived transconjugants carrying MBL-plasmids produced less biofilm but showed higher virulence to larvae than recipient AB218.

Conclusions: Our 19-year longitudinal study revealed a stable increase in CNSA during 2005—2015. *bla*$_{OXA-24-like}$, IS*Aba1*-*bla*$_{OXA-51-like}$, and IS*Aba1*-*bla*$_{OXA-23}$ were the major determinants of *Acinetobacter* carbapenem resistance. MBL-carrying plasmids contribute not only to the carbapenem resistance but also to *A. baumannii* virulence.

Introduction

Acinetobacter infections are predominantly caused by members of the *Acinetobacter calcoaceticus-baumannii* complex (ACB complex), including *A. baumannii*, *A. nosocomialis*, and *A. pittii*.[1] Infections caused by *A. baumannii* are often severe and difficult to treat due to high rates of resistance to antibiotics.[2] Furthermore, high proportions of *Acinetobacter* isolates are now nonsusceptible to the most widely used antimicrobial agents, including carbapenems.[3] Carbapenems were previously considered the most efficient β-lactam antibiotics in treating various bacterial infections due to their broad spectrum characteristics and relative resistance to hydrolysis by most β-lactamases.[4] The resistance rate to carbapenems in clinical *A. baumannii* isolates has increased to more than 50% in Asia, Latin America, and Europe.[5,6]

In addition to porin CarO dysfunction and efflux pump overexpression,[7,8] carbapenemase-encoding genes carried on the *A. baumannii* chromosome and/or plasmids are often associated with mobile genetic elements (including insertion sequences (IS), integrons, and transposons) and contribute to carbapenem hydrolysis and resistance. Carbapenemases can be divided into metallo-β-lactamases (MBL, class B β-lactamases, including *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{NDM}$) and non-metallo-β-lactamases (class A and D β-lactamases).[9] Among the carbapenemases, oxacillinases (OXAs, class D β-lactamases) are the most common carbapenem resistance genes identified in *Acinetobacter* isolates.[9]

The prevalence and characteristics of carbapenem-nonsusceptible *Acinetobacter* (CNSA) isolates have been reported worldwide; however, the isolates in most studies were enrolled for a relatively short period. Longitudinal surveillance to study epidemiologic trends and characteristics of CNSA isolates is possibly hidden. Therefore, this study aimed to characterize CNSA isolated from patients with bloodstream infections in a university hospital in Taiwan during 1997—2015 and to determine the role(s) of conjugative plasmids carrying MBL in the virulence of *Acinetobacter*.

Methods

Sampling and isolation of *Acinetobacter*

The *Acinetobacter* isolates were recovered from patients with bacteremia at National Cheng Kung University Hospital (NCKUH), from 1997 to 2015. *Acinetobacter* isolates were identified in the clinical laboratory by colony morphology, Gram stain, biochemical tests, and the Vitek system (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's recommendations. A total of 1403 non-duplicate clinical isolates were collected and stored at −80 °C in tryptic soy broth (TSB) containing 20% glycerol (v/v) until use.

Antimicrobial susceptibility testing

To identify CNSA isolates, the susceptibility to imipenem of 1403 *Acinetobacter* isolates was determined by the disk diffusion method and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[10] The minimal inhibitory concentration (MIC) values for imipenem and meropenem were further determined by broth dilution tests in 173 CNSA isolates according to the CLSI guidelines to validate disk diffusion results.[10] *Acinetobacter* isolates with imipenem or meropenem MIC ≥4 μg/mL were defined as carbapenem nonsusceptible.[10] Antimicrobial susceptibility of 173 CNSA isolates to 12 antibiotics was determined by disk diffusion assay. Isolates were subcategorized as multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR), according to the previous study.[11]

Species identification

A previously described multiplex PCR-based assay was first performed to identify the species of CNSA isolates.[12] The PCR primers used in this study are described in Table S1.

Three pairs of primers targeting *recA* and *gyrB* genes and the 16S-23S rDNA intergenic spacer region were used to differentiate *A. baumannii*, *A. nosocomialis*, *A. pittii*, and other *Acinetobacter* species. Non-ACB complex isolates were identified at the species level using 16S rDNA sequence analysis as previously described.[13]

Detection of β-lactamase genes and transposons

All CNSA isolates were further tested for the presence of oxacillinase genes ($bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$, $bla_{OXA\text{-}143}$, and $bla_{OXA\text{-}235}$), upstream location of insertion sequences (ISs) of oxacillinase genes (IS*Aba1*-$bla_{OXA\text{-}51\text{-}like}$, IS*Aba1*-$bla_{OXA\text{-}23\text{-}like}$, and IS*1008*, IS*1006* or IS*15DI* upstream of $bla_{OXA\text{-}58\text{-}like}$), and selected β-lactamase genes (bla_{DIM}, bla_{GES}, bla_{IMI}, bla_{SME}, bla_{GIM}, bla_{IMP}, bla_{KPC}, bla_{NDM}, bla_{SIM}, bla_{SPM}, and bla_{VIM}) by PCR amplification with specific primers, as previously described.[14] The PCR primers are described in Table S1.

Conjugation experiments and plasmid analysis

The liquid mating-out assay was performed to determine the transferability of plasmid-carrying MBL genes from isolates to rifampicin-resistant *E. coli* C600, *A. baumannii* 218 (AB218), and *A. nosocomialis* 254 (AN254), as previously described.[15] The transconjugants were selected on LB plates containing 256 μg/mL rifampicin (*E. coli* C600) (Sigma) or 256 μg/mL amikacin (AB218 and AN254) (Sigma) combined with 4 μg/mL meropenem (Sigma). PCR was performed to confirm the presence of bla_{VIM} and bla_{IMP} in the transconjugants. Random amplified polymorphic DNA (RAPD)-PCR with primers M13 and DAF4 was used to verify that the transconjugants were derived from recipients. The primers used for detecting carbapenemase genes and RAPD-PCR are listed in Table S1.

Genome sequencing, assembly, annotation, and analysis

The whole genome sequence of the isolates tested was determined by Nanopore genome sequencing platform, and the genome was constructed with Flye *de novo* assembler (version 2.9).[16,17] Genome annotation was performed by NCBI Prokaryotic Genome Annotation Pipeline (PGAP, version 6.1) (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). ResFinder (http://www.genomicepidemiology.org/) was used to find the antibiotic resistance genes.

Genome comparison and phylogenetic analysis of closely related plasmids of pAS566-1

BLAST Ring Image Generator (BRIG) (version 0.95) was used to generate images showing plasmid comparisons. The assembled plasmid sequence was submitted to BLAST and compared with previously sequenced plasmids deposited in the NCBI GenBank database. Fourteen plasmids with a higher maximum score and a higher degree of similarity (>80% query coverage and >90% identity) to the plasmid sequence of pAS566—1 were selected for further analysis. CulstalW alignment was performed to construct Neighbor-Joining trees inferred with the Jukes-Cantor model and 500 bootstrap replicates by using Molecular Evolutionary Genetics Analysis (MEGA) software version 11.

Biofilm formation assay

Biofilm formation was assessed using a 96-well flat-bottom polystyrene microtiter plate, as previously described,[17] with minor modifications. Biofilms were stained with 0.1% crystal violet for 30 min. Crystal violet dye associated with biofilms was eluted with 95% ethanol for 30 min and was quantified by absorbance at 590 nm.

Iron acquisition determined by chrome azurol S agar assay

Chrome azurol S (CAS) agar plates were prepared as previously described.[18] The modified CAS agar plate was punched with 6.5-mm diameter holes by using a tip (Corning® Pasteur pipettes, no. L, 5 3/4 inch (146 mm)). Each hole was filled with 20 μL of the LB broth containing an equal colony-forming unit (CFU) of bacteria, incubated at 37 °C, and monitored for the formation of an orange halo until 72 h. CAS remains blue when complexed with iron but turns orange when iron is chelated by other iron chelators. The siderophore activity was expressed as the diameter of the halo.

Galleria mellonella larvae infection

The *Galleria mellonella* larvae infection test was carried out as previously reported to evaluate bacterial virulence.[19,20] The larvae weighed around 220 mg were used in this assay. Overnight bacterial cultures established in LB were washed and resuspended in PBS at an OD_{600} of 1 and then adjusted to 1.7×10^7 CFU/mL. Each set of ten larvae received a 10 μL bacterial suspension injection into the second to last left proleg, while control larvae received the same volume of sterilized PBS injection. Larvae were kept at 37 °C in the dark without feeding for seven days, and survival was recorded every 24 h until seven days after injection. This assay used the recipient strain of transconjugants as a negative control. The larvae infection was performed in biological duplicates to ensure reproducibility (20 larvae/strain).

Statistical analysis

Data were expressed as a mean of three replicates ± SD. One-way ANOVA test was used to calculate the statistical significance of the experimental results between the two groups. The two-way ANOVA test was used to assess multiple comparisons in the groups in the iron acquisition assay. Mantel-Cox was used to compare the larval survival rate between the two groups. All statistical analyses were conducted using GarphPad Prism version 8.0.2. A *p*-value less than 0.05 was considered a significant difference.

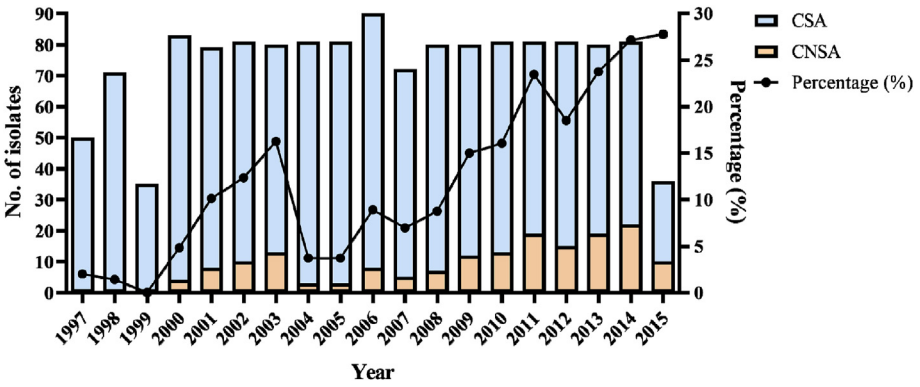


Figure 1. Prevalence of carbapenem-nonsusceptible *Acinetobacter* isolated from patients with bloodstream infections in Taiwan, 1997–2015. The number of carbapenem-susceptible *Acinetobacter* (CSA) and carbapenem-nonsusceptible *Acinetobacter* (CNSA) isolates is plotted as bars on the primary axis while the percentage of CNSA isolates is plotted as a line graph on the secondary axis.

Results

Stable increase in carbapenem-nonsusceptible *Acinetobacter* in Taiwan

A total of 173 CNSA (12.3%) isolates were identified from 1403 *Acinetobacter* isolates recovered from patients with bloodstream infections at NCKUH from 1997 to 2015. The results showed an increasing prevalence of CNSA strains isolated from patients with bacteremia during 1999–2003 and 2005–2015; however, a dramatic decrease in the annual prevalence of CNSA was observed in 2004 (3/81, 3.7%) and 2005 (3/81, 3.7%) (Fig. 1). Only one CNSA strain was found in 50 *Acinetobacter* isolates in 1997 (2.0%). On the contrary, 27.8% (10/36) of *Acinetobacter* strains isolated in 2015 were CNSA (Fig. 1).

We further performed multiplex PCR to determine the distribution of *Acinetobacter* species in 173 CNSA isolates. The results showed that *A. baumannii* (95 isolates, 54.9%) is the most common detected species, followed by *A. nosocomialis* (40 isolates, 23.1%), unspecified (37 isolates, 21.4%), and *A. pittii* (1 isolate, 0.6%). 16S rDNA PCR-sequencing was performed on 37 unspecified strains to determine the species of these strains, and the results revealed 21 *A. soli*, 6 *A. johnsonii*, 5 *A. bereziniae*, 1 *A. junii*, 1 *A. higginsii*, 1 *A. bouvetii*, 1 *A. lowffii*, and 1 *A. ursingii* (Table S2).

We determined the antimicrobial susceptibility of CNSA isolates to other 12 antimicrobials (divided into eight categories). Then, we classified our isolates into non-MDR-, MDR-, XDR-, or PDR-isolates (Table S2), according to the previous study.[11] We found 167 MDR-CNSA (96.5%) and 49 XDR-CNSA (28.3%) (Table S2). All XDR-CNSA isolates were *A. baumannii*. Furthermore, 93 (97.9%) and 49 (51.6%) carbapenem-nonsusceptible *A. baumannii* were MDR and XDR, respectively (Table S2). However, no PDR-CNSA was identified in our 173 CNSA isolates.

PCR detection of genes encoding carbapenemases and insertion sequences

Carbapenemase genes, including bla_{DIM}, bla_{GES}, bla_{IMI}, bla_{SME}, bla_{GIM}, bla_{KPC}, bla_{NDM}, bla_{SIM}, and bla_{SPM}, were not detected in our 173 CNSA isolates. Metallo-β-lactamases, bla_{VIM-11}, bla_{IMP-1}, and bla_{IMP-19} were detected in 9 (5.2%), 20 (11.6%), and 1 (0.6%) CNSA isolates, respectively (Fig. 2).

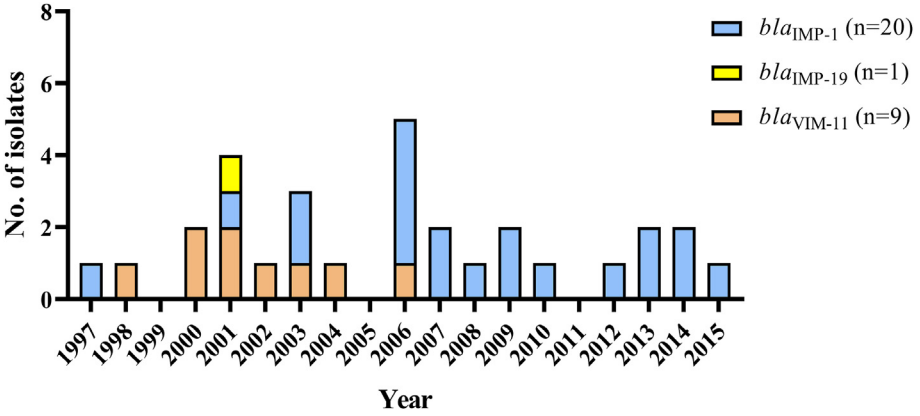


Figure 2. The distribution of bla_{IMP-1}, bla_{IMP-19}, and bla_{VIM-11} in CNSA isolates during 1997 to 2015. In 2001, only one isolate containing bla_{IMP-19} was identified, and 20 isolates contained bla_{IMP-1}. bla_{VIM-11} was identified in nine CNSA isolates.

bla$_{VIM-11}$ was identified in CNSA isolates between 1998 and 2006, and only one isolate containing *bla*$_{IMP-19}$ was identified in 2001 (Fig. 2). The distribution of *bla*$_{IMP-1}$ was dispersed between 1997 and 2015. *bla*$_{IMP-19}$ was detected in an *A. baumannii* isolate, and *bla*$_{IMP-1}$ was detected in 6, 10, 1, 1, 1, and 1 isolates of *A. johnsonii*, *A. soli*, *A. nosocomialis*, *A. junii*, *A. ursingii*, and *A. bouvetii*, respectively. *bla*$_{VIM-11}$ was detected in 3, 5, and 1 isolates of *A. soli*, *A. bereziniae*, and *A. higginsii*, respectively (Table S3). One *A. bereziniae* isolate 363 had *bla*$_{VIM-11}$ showed sensitivity to meropenem (2 μg/mL) and low resistance to imipenem (16 μg/mL) (Table S3).

The detection of OXA-type carbapenemase gene-associated genetic structures revealed that *bla*$_{OXA-24-like}$ (40 isolates, 23.1%) dominated in 173 CNSA isolates, followed by IS*Aba1*-*bla*$_{OXA-23}$ (30 isolates, 17.3%), IS*Aba1*-*bla*$_{OXA-51}$ (24 isolates, 13.9%), IS*1006*-*bla*$_{OXA-58}$+IS*1008*-*bla*$_{OXA-58}$ (16 isolates, 9.2%), IS*Aba1*-*bla*$_{OXA-51}$+*bla*$_{OXA-24}$ (10 isolates, 5.8%), IS*Aba1*-*bla*$_{OXA-23}$+*bla*$_{OXA-24}$ (4 isolates, 2.3%), and IS*1008*-*bla*$_{OXA-58}$ (2 isolates, 1.2%) (Table 1). Moreover, *bla*$_{OXA-24}$+*bla*$_{IMP-1}$, *bla*$_{OXA-24}$+*bla*$_{VIM-11}$, IS*1006*-*bla*$_{OXA-58}$, IS*Aba1*-*bla*$_{OXA-23}$+IS*Aba1*-*bla*$_{OXA-51}$, and IS*Aba1*-*bla*$_{OXA-51}$+IS*1008*-*bla*$_{OXA-58}$, each was detected in only one CNSA isolate (Table 1). The gene encoding *bla*$_{OXA-143}$ and *bla*$_{OXA-235}$ carbapenemase was not detected in our 173 CNSA isolates.

Among 95 carbapenem-nonsusceptible *A. baumannii* isolates, *bla*$_{OXA-24}$, IS*Abal*-*bla*$_{OXA-23}$, and IS*Aba1*-*bla*$_{OXA-51}$ were found in 29 (30.5%), 28 (29.5%), and 23 (24.2%) isolates, respectively (Table 1). In contrast, IS*1006*-*bla*$_{OXA-58}$+IS*1008*-*bla*$_{OXA-58}$ (15 isolates, 37.5%), *bla*$_{OXA-24}$ (11 isolates, 27.5%), and IS*Aba1*-*bla*$_{OXA-51}$+*bla*$_{OXA-24}$ (9 isolates, 22.5%), were dominant in 40 carbapenem-nonsusceptible *A. nosocomialis* isolates. *bla*$_{IMP}$ (19 isolates, 50.0%) and *bla*$_{VIM-11}$ (9 isolates, 23.7%) were prevalent in CNSA isolates from other *Acinetobacter* species (Table 1).

Efflux pump activity and CarO variants in CNSA isolates without *bla*$_{OXA}$ overexpression and metallo-β-lactamase

We found that 14 CNSA isolates (7 *A. baumannii* and 7 *A. soli*) did not have *bla*$_{OXA-24}$, *bla*$_{OXA}$ overexpression, and MBL genes but had a MIC to imipenem ranging from 16 to 256 μg/mL (Table S4). Therefore, we determined the contribution of efflux pumps to carbapenem resistance in these 14 CNSA isolates. The effect of efflux pump inhibitor CCCP to suppress the growth of *Acinetobacter* was first evaluated; however, the results showed that 25 μg/mL CCCP used in the previous study[21] suppressed the growth of our tested isolates. All isolates tested grew well under treatment with 10 μg/mL CCCP, and this concentration was employed for the efflux pump inhibition assay. However, only isolate 1319 showed a 4-fold decrease in MIC (64—16 μg/mL) to imipenem in combination with 10 μg/mL CCCP (Table S4).

The CarO amino acid sequences of these 14 isolates were compared with the CarO sequence of carbapenem-susceptible *A. baumannii* AB307-0294 (type III CarO), *A. nosocomialis* WM98B (type I CarO), and *A. baumannii* 17978 (untypable CarO). Five and two *A. baumannii* isolates had type III and type I CarO, respectively. Four *A. soli* isolates had type III CarO. However, no *carO* PCR product with the expected size was detected in three *A. soli* isolates (Table S4). All nine isolates with type III CarO contained only a T214S amino acid substitution compared to CarO of *A. baumannii* AB307-0294 (Table S4). To delineate whether type III CarO containing a T214S amino acid substitution is associated with carbapenem resistance, we examined the CarO sequence of two carbapenem-susceptible *A. baumannii* (isolates 263 and 264). The results showed that the substitution for CarO T214S was also found in isolates 263

Table 1 The distribution of carbapenemase genes, *Acinetobacter* species, and MIC ranges to imipenem and meropenem of 173 carbapenem non-susceptible *Acinetobacter* isolates.

Carbapenemase gene	*Acinetobacter* species, n (%)				MIC range (μg/mL)	
	A. baumannii (n = 95)	*A. nosocomialis* (n = 40)	other *Acinetobacter* spp. (n = 38)	Total (n = 173)	MEM	IPM
bla$_{IMP-1/19}$	1 (1.1)[a]	1 (2.5)	18 (47.4)	20 (11.6)	4—256	8 - >256
bla$_{VIM-11}$	0 (0)	0 (0)	8 (21.1)[b]	8 (4.6)	2—256	8—128
bla$_{OXA-24}$	29 (30.5)	11 (27.5)	0 (0)	40 (23.1)	16—256	16—256
bla$_{OXA-24}$+*bla*$_{IMP-1}$	0 (0)	0 (0)	1 (2.6)	1 (0.6)	32	256
bla$_{OXA-24}$+*bla*$_{VIM-11}$	0 (0)	0 (0)	1 (2.6)	1 (0.6)	4	16
IS*1006*-*bla*$_{OXA-58}$	0 (0)	0 (0)	1 (2.6)	1 (0.6)	4	16
IS*1008*-*bla*$_{OXA-58}$	1 (1.1)	0 (0)	1 (2.6)	2 (1.2)	16	32
IS*1006*-*bla*$_{OXA-58}$+IS*1008*-*bla*$_{OXA-58}$	1 (1.1)	15 (37.5)	0 (0)	16 (9.2)	8—32	4—32
IS*Aba1*-*bla*$_{OXA-23}$+*bla*$_{OXA-24}$	3 (3.2)	1 (2.5)	0 (0)	4 (2.3)	128—256	256
IS*Aba1*-*bla*$_{OXA-23}$	28 (29.5)	1 (2.5)	1 (2.6)	30 (17.3)	4—256	16—256
IS*Aba1*-*bla*$_{OXA-23}$+IS*Aba1*-*bla*$_{OXA-51}$	1 (1.1)	0 (0)	0 (0)	1 (0.6)	128	128
IS*Aba1*-*bla*$_{OXA-51}$+*bla*$_{OXA-24}$	1 (1.1)	9 (22.5)	0 (0)	10 (5.8)	128—256	128—256
IS*Aba1*-*bla*$_{OXA-51}$	23 (24.2)	1 (2.5)	0 (0)	24 (13.9)	4—256	16—256
IS*Aba1*-*bla*$_{OXA-51}$+IS*1008*-*bla*$_{OXA-58}$	0 (0)	1 (2.5)	0 (0)	1 (0.6)	16	32
Total (n, (%))	88 (92.6)	40 (100)	31 (81.6)	159 (91.9)	—	—

[a] Only one *A. baumannii* isolate had *bla*$_{IMP-19}$.
[b] One *A. bereziniae* isolate had *bla*$_{VIM-11}$ that showed susceptibility to meropenem (2 μg/mL) and relatively low resistance to imipenem (16 μg/mL).
IPM, imipenem; MEM, meropenem.

and 264. These results suggest that the single amino acid variant T214S in Type III CarO is not associated with the carbapenem resistance of *Acinetobacter*. Moreover, two carbapenem-nonsusceptible *A. baumannii* isolates (535 and 1648) had wild-type Type I CarO (Table S4).

Transferability and whole genome sequence of plasmids carrying metallo-β-lactamases genes

We then investigated the transferability of MBL-carrying plasmids to *A. baumannii* (AB218), *A. nosocomialis* (AN254), and *E. coli* (C600) using conjugation tests (Table S3). Transfer of MBL genes by conjugation to recipient cells of AB218 and AN254 was successful for 7 and 6 of the 30 MBL-carrying plasmids, respectively (Table S3). A conjugative bla_{VIM-11} was only identified in plasmid pAS435-1. Moreover, four plasmids carrying bla_{IMP-1} from the parental isolates *A. johnsonii* 54, *A. soli* 566, *A. soli* 843, and *A. soli* 967—1, were successfully transferred to both AB218 and AN254 (Table S3). However, all plasmids carrying MBL genes could not be transferred to the recipient *E. coli* C600 strain. The MBL genes in the transconjugants were further validated by PCR, and all transconjugants were not susceptible to carbapenems.

The Nanopore whole genome sequencing was then performed on nine CNSA isolates that harbor conjugative MBL-plasmids listed in Table S3 to characterize the plasmids further. The WGS results showed that the range of GC content was 38.7—40.1%, contained 286 to 385 open reading frames, and the plasmid sizes ranged from 248,123 bp (pAJ54-1) to 325,311 bp (pAS566-1) (Table S5). Therefore, pAS566-1, which had the largest plasmid size, was used as a control sequence compared to the other eight plasmids. The results showed that pAS435-1 plasmid contained bla_{VIM-11} and the other eight plasmids contained bla_{IMP-1}; however, all these plasmids shared a very similar sequence backbone (Fig. 3A). There are still some differences between pAS566-1 and the other eight plasmids (white gaps in Fig. 3A). These gaps might be due to the integration of mobile elements or recombination of plasmids. Thus, these results revealed a high recombination frequency in these plasmids (Fig. 3A).

The plasmid BLAST results showed that the sequence of pAS566-1 was very similar to plasmids *A. soli* strain AS843 plasmid pAS843-1 (accession number CP119204.1, coverage 100%, identity 99.97%), *A. soli* strain AS967-1 plasmid pAS967-1-1 (accession number CP119216.1, coverage 100%, identity 99.98%), *A. soli* strain AS903-1 plasmid pAS903-1-1 (accession number CP119208.1, coverage 97%, identity 99.98%), *A. bereziniae* strain AB839 plasmid pAB839-1 (accession number CP119243.1, coverage 92%, identity 99.91%), and *A. pittii* strain 2014N21-145 plasmid p2014N21-145-1 (accession number CP033569.1, coverage 88%, identity 99.26%), *A. johnsonii* XBB1 plasmid pXBB1-9 (accession number CP010351.1, coverage 88%, identity 98.81%), *Acinetobacter* sp. WCHA55 plasmid pOXA58_010055 (accession number CP032285.1, coverage 85%, identity 99.73%), *Acinetobacter defluvii* strain WCHA30 plasmid pOXA58_010030 (accession number CP029396.2, coverage 85%, identity 99.72%), *Acinetobacter seifertii* strain AS4 plasmid pAS4-1 (accession number CP061688.1, coverage 83%, identity 99.95%), *A. soli* strain AS435 plasmid pAS435-1 (accession number CP119229.1, coverage 82%, identity 99.86%), *A. soli* strain AS645 plasmid pAS645-1 (accession number CP119239.1, coverage 82%, identity 99.92%), *A. johnsonii* strain Acsw19 plasmid pAcsw19-2 (accession number CP043309.1, coverage 82%, identity 99.72%), *Acinetobacter ursingii* strain RIVM0061 plasmid pRIVM0061_IMP-4_171109_B01 (accession number MH220287.1, coverage 80%, identity 99.69%), and *A. ursingii* strain RIVM_C010559 plasmid pRIVM_C010559_1 (accession number CP089052.1, coverage 80%, identity 99.69%) (Table S6).

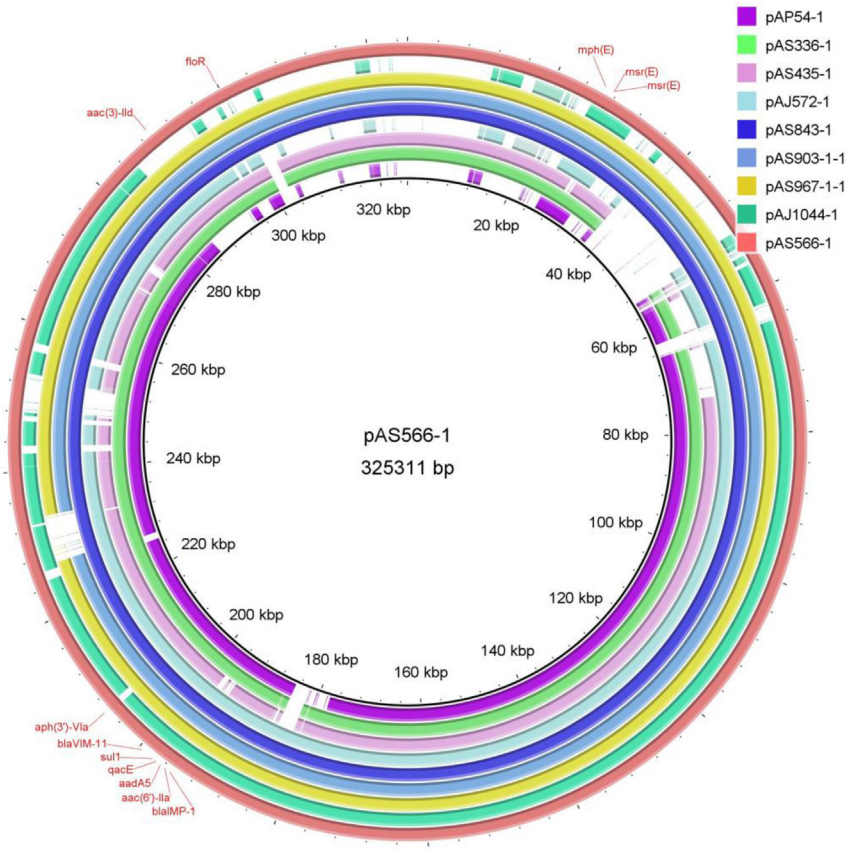
The plasmids pXBB1-9, pOXA58_010055, pOXA58_010030, and pAcsw19-2, were identified in China, the plasmids pRIVM0061_IMP-4_171109_B01 and pRIVM_C010559_1 were identified in the Netherlands, and the other nine plasmids were identified in Taiwan. Importantly, plasmids pOXA58_010055, pOXA58_010030, and pAS4-1 did not contain MBL, and bla_{IMP-1}, bla_{IMP-4}, bla_{VIM-11}, and bla_{NDM-1}, were detected in five, two, three, and two plasmids, respectively (Table S6). The phylogenetic tree indicated that pAS566-1 showed the highest similarity to the plasmid pAS903-1-1, followed by pAS967-1 and pAS843-1 in *A. soli* (Fig. 3B).

MBL-carrying plasmids are associated with biofilm formation, iron acquisition, and *Acinetobacter* virulence to larvae

To characterize the biological function of MBL-carrying plasmids in *Acinetobacter* in addition to antibiotic resistance, we compared virulence-associated phenotypes, including biofilm formation, iron acquisition, and virulence to larvae, between recipients and transconjugants (Fig. 4). The results of biofilm formation in LB (Fig. 4A) and M9 (nutrition-limited medium) (Fig. 4B) showed that all transconjugants had a significant decrease in biofilm formation after two days of incubation, compared to the recipient AB218. In contrast, transconjugants having plasmids pAS336-1, pAS843-1, and pAS967-1-1 showed a significant decrease in biofilm formation in LB (Fig. 4A), and transconjugants having plasmids pAP54-1, pAS336-1, pAS566-1, and pAS967-1-1 showed a significant decrease in biofilm formation in M9 (Fig. 4B), compared to recipient AN254. There was no significant difference in iron acquisition of seven AB218-derived transconjugants compared to the recipient AB218. However, all six AN254-derived transconjugants had a significant decrease in their iron acquisition, compared to the recipient AN254 (Fig. 4C).

We further tested the contribution of MBL-carrying plasmids to bacterial virulence using the larvae infection model (Fig. 4D). Larval survival rate was determined over a 7-day time course for this analysis, and the negative control group for PBS injection did not show fatalities. The larvae were considered dead when they failed to respond to touch. On day seven after infection, larvae infected with AB218 had a survival rate of 60% (Fig. 4D). The seven AB218-derived transconjugants that carried MBL-plasmids showed higher virulence to larvae with a low larvae survival rate compared to the recipient AB218 (Fig. 4D). However, MBL-

(A).



(B).

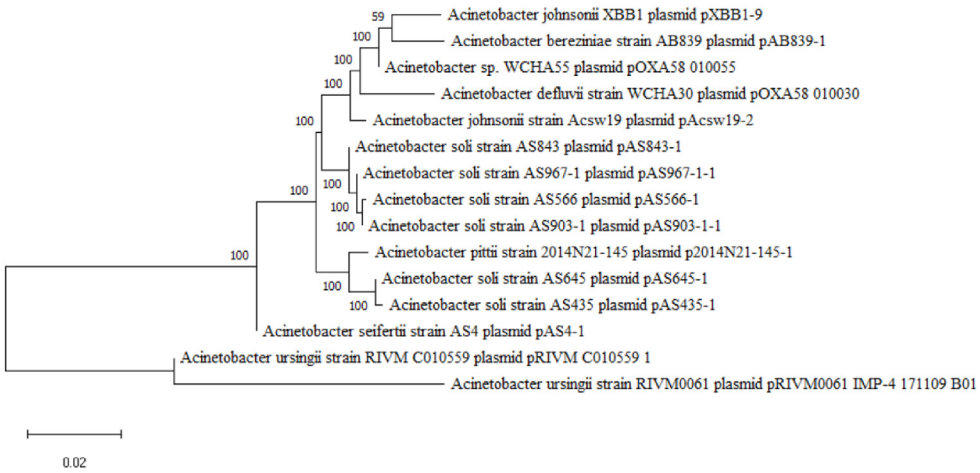


Figure 3. Genomic comparison of plasmids closely related to pAS566−1. (A). Blast Ring Image Generator (BRIG) diagram shows homologous segments of eight conjugative MBL-carrying plasmids with pAS566-1 as a reference. Antibiotic resistance genes in pAS566-1 were indicated. bla_{VIM-11} was found only in pAS435-1. **(B).** The phylogenetic tree was built based on the aligned nucleotide sequences of 15 plasmids by using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

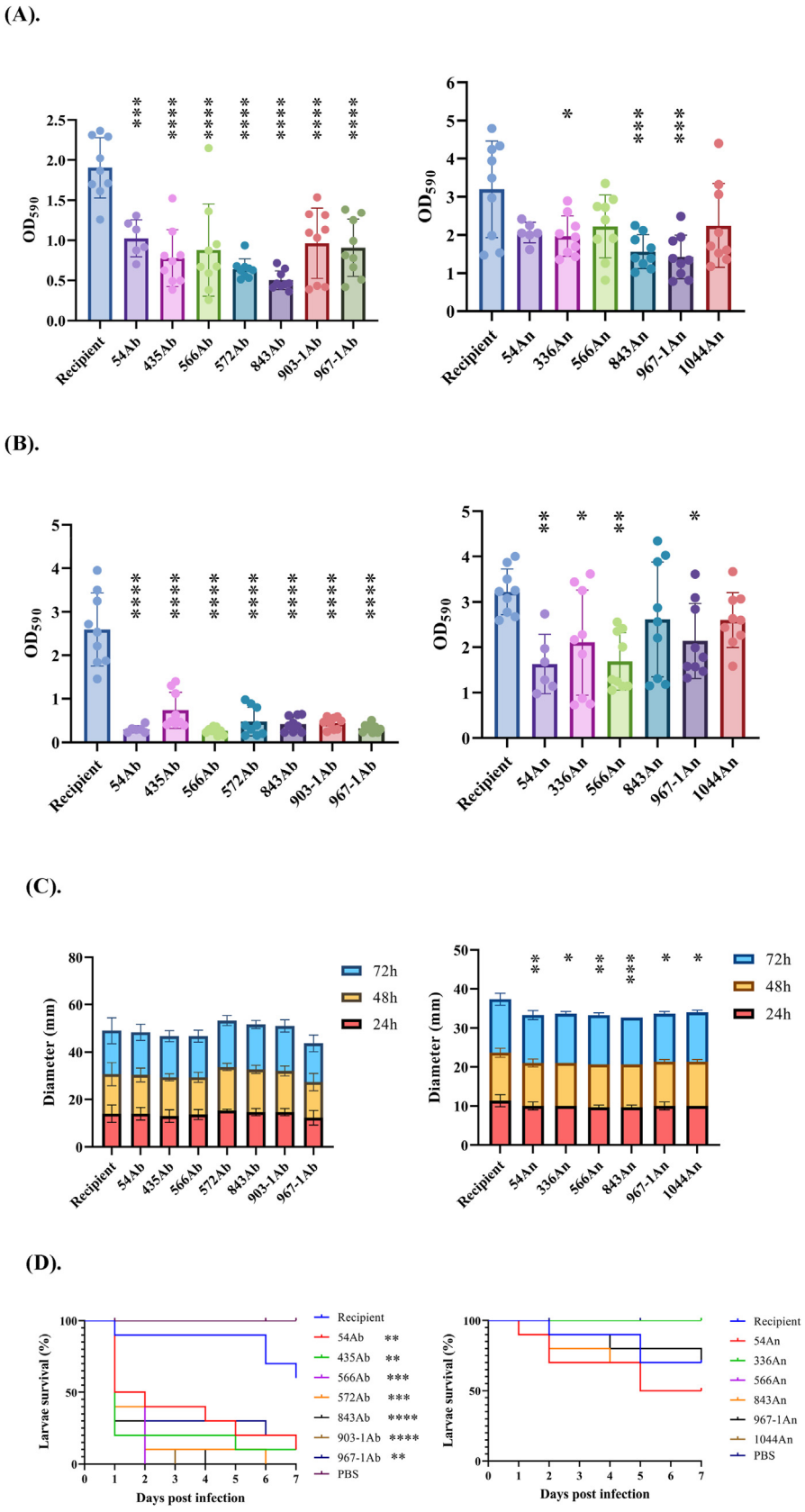


Figure 4. Characterization of phenotypes of transconjugants derived from recipients *A. baumannii* AB218 and *A. nosocomialis* AN254. Biofilms of bacteria in LB **(A)** and M9 broth **(B)** were stained with crystal violet and measured at 590 nm. **(C).** Iron acquisition activity was determined by CAS agar assay. The halo diameter representing the iron acquisition activity was measured after 24, 48, and 72 h of incubation. **(D).** *Galleria mellonella* survival curves for seven days when larvae infected with the strains

carrying plasmids did not contribute to the virulence of recipient AN254 (Fig. 4D).

Discussion

Although carbapenems have been considered critical antimicrobials in treating MDR *Acinetobacter* infections, a rapid increase in CNSA has been observed worldwide. Here, we present a 19-year longitudinal collection of CNSA isolated from patients with bacteremia to investigate carbapenem resistance mechanisms in *Acinetobacter* isolates. Our results showed a generally stable increase in isolated CNSA strains from 1999 to 2015, but a relatively low prevalence of CNSA during 2004–2008 was observed compared to 2003 (Fig. 1). Antimicrobial stewardship programs in Taiwanese hospitals from early 2000 seek to optimize antimicrobial prescribing to improve individual patient care, reduce hospital costs, and slow the spread of antimicrobial resistance, which can lead to the relatively low prevalence of CNSA during 2004–2008. However, the spread of CNSA clones and the plasmid-mediated resistance may contribute to the increase in CNSA incidences after 2008.

Although antibiotic options preferred for the treatment of carbapenem-resistant *A. baumannii* infections including polymycin B, minocycline, tigecycline, cefiderocol, ampicillin-sulbactam, and sulbactam-durlobactam,[22] our results detected that 78 (45.1%) and 8 (4.6%) CNSA were not susceptible to ampicillin-sulbactam and tigecycline, respectively (data not shown). However, all 173 CNSA were susceptible to colistin. Although the susceptibility of our CNSA to polymycin B, minocycline, cefiderocol, and sulbactam-durlobactam is unclear, our results suggest that tigecycline and colistin can be used as antibiotics recommended for the treatment of CNSA in Taiwan.

We found that $bla_{\text{OXA-24-like}}$, IS*Aba1*-$bla_{\text{OXA-23}}$, IS*Aba1*-$bla_{\text{OXA-51-like}}$, and bla_{IMP} were the major carbapenem resistance determinants, and the distribution of these determinants was associated with *Acinetobacter* species (Table 1). The MBL gene is primarily detected in strains other than *A. baumannii* and *A. nosocomialis*. Therefore, the primary cause for carbapenem non-susceptibility in *A. baumannii* isolates is the presence $bla_{\text{OXA-24-like}}$, IS*Aba1*-$bla_{\text{OXA-23}}$, and IS*Aba1*-$bla_{\text{OXA-51-like}}$. In carbapenem non-susceptible *A. nosocomialis*, it is due to isolates carrying $bla_{\text{OXA-24-like}}$, IS*1006*-$bla_{\text{OXA-58}}$+IS*1008*-$bla_{\text{OXA-58}}$, and IS*Aba1*-$bla_{\text{OXA-51}}$+$bla_{\text{OXA-24}}$, leading to their carbapenem non-susceptibility (Table 1). Additionally, we observed a low transferability of MBL-carrying plasmids to AB218 (23.3%) and AN254 (20.0%) of the 30 MBL-carrying plasmids (Table S3). These results suggest a conjugative barrier for MBL-carrying plasmids from non-*A. baumannii* and non-*A. nosocomialis* isolates to *A. baumannii* and *A. nosocomialis*.

Four MBL-carrying plasmids, pAP54-1, pAS566-1, pAS843-1, and pAS967-1-1, were transferable to *A. baumannii* and *A. nosocomialis*, the high similarity of the plasmid sequences was observed between pAS566-1, pAS843-1, and pAS967-1-1, and the antimicrobial resistance determinants were identical in these three isolates (Table S6). CNSA isolates 566, 843, and 967, were isolated in 2003, 2006, and 2007, respectively. Therefore, the circulation of specific MBL-carrying plasmids of *Acinetobacter* should be monitored.

The dominant MBL genes identified in this study were $bla_{\text{VIM-11}}$ and $bla_{\text{IMP-1}}$. The results were consistent with Lin's report.[23] Interestingly, no VIM-11-producing *Acinetobacter* was identified after 2006 in our collection. Eight out of 21 bla_{IMP}-producing isolates showed the transferability of bla_{IMP} to *A. baumannii* and/or *A. nosocomialis*. In contrast, conjugation tests showed that even though the nine plasmids carried $bla_{\text{VIM-11}}$, only the plasmid pAS435-1 was transferable to *A. baumannii*, which may limit the spread of plasmids carrying $bla_{\text{VIM-11}}$ in the environment. Moreover, we found that $bla_{\text{VIM-11}}$ was only identified in *A. bereziniae* (5 isolates), *A. soli* (3 isolates), and *A. higginsii* (1 isolate). To our knowledge, this is the first report to identify the presence of $bla_{\text{VIM-11}}$ in *A. soli*, *A. higginsii*, and *A. bereziniae* in Taiwan.

To determine carbapenem resistance mechanisms in 14 CNSA isolates without $bla_{\text{OXA-24}}$, bla_{OXA} overexpression, and class B carbapenemase, we examined *carO* in these strains. We found nine isolates with type III CarO contained a T214S amino acid substitution compared to the CarO of *A. baumannii* AB307-0294. Although the substitution of CarO T214S amino acid located between β-sheet 7 and 8 was also identified in three carbapenem-resistant *A. baumannii* isolates (B1, B4, and B7) by Labrador-Herrera et al.,[24] the CarO of two carbapenem susceptible *A. baumannii* also had a T214S substitution in this study. These results suggest that the T214S substitution may not involved in carbapenem resistance in *Acinetobacter*. *carO* was not detected in three carbapenem-resistant *A. soli* isolates (1261–1, 1301, and 1307) containing $bla_{\text{OXA-58}}$ (Table S4). Lee et al. reported that the insertion sequence, IS*Aba10*, was inserted not only into IS*Aba1* adjacent to the $bla_{\text{OXA-23}}$, but also disrupted the *carO* in *A. baumannii*.[25] Therefore, these results suggest the incomplete structure of the *carO* gene in these three *A. soli* isolates and thus cause resistance to carbapenem. Reduced CarO expression contributes to *Acinetobacter* resistance to carbapenem[7]; therefore, the level of CarO expression in these CNSA isolates with wild-type *carO* is worth investigating. Moreover, resistance to imipenem in *A. baumannii* is linked to reduced expression of a 33–36 kDa outer membrane protein.[26] Conversely, the influence of OmpA on antibiotic resistance is particularly significant in gene disruption mutants, as they display increased susceptibility to nalidixic acid, chloramphenicol, aztreonam, imipenem, and meropenem.[27] Therefore, for isolates in which the relevant carbapenem resistance mechanism can not be identified in this study, it remains to be clarified whether other OMP structures and expressions, apart from CarO, also impact resistance development.

In addition to carbapenemase and CarO dysfunction, efflux pump overexpression is associated with decreased carbapenem susceptibility in *Acinetobacter*. We

tested. The inoculum for bacteria was 1.7×10^5 CFU/larvae. For each experiment, 10 larvae per strain were used to be tested. All experiments were performed in biological triplicate. The error bars represent the standard deviations of three independent experiments. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

determined the contribution of efflux pumps in our 14 CNSA isolates listed in Table S4. However, a concentration of CCCP higher than 10 μg/mL suppressed the growth of our *Acinetobacter*, and only isolate 1319 showed a 4-fold change in MIC to imipenem in combination with 10 μg/mL CCCP. These results suggest that low-concentration CCCP (10 μg/mL) may not inhibit efflux pump activity completely. Furthermore, consistent with Zhu's report,[28] these results suggest that CCCP is not a suitable inhibitor for detecting the antimicrobial-resistance effect of efflux pumps on *Acinetobacter*. The other efflux pump inhibitor, Phenylalanine-Arginine β-Naphthylamide (PAβN), showed a 4 to 64-fold reduction in the MIC for 58 of 60 isolates of *A. baumannii*.[29] Therefore, whether efflux pumps contribute to carbapenem resistance in our CNSA isolates remains to be clarified by using other inhibitors.

Among our 173 CNSA isolates, *A. baumannii* (54.9%) is the most common species detected, followed by *A. nosocomialis* (23.1%), *A. soli* (12.1%), *A. johnsonii* (3.5%), and *A. bereziniae* (2.9%). Endo et al. reported a high frequency of *A. soli* among *Acinetobacter* strains isolated from patients with bacteremia in Japan.[30] They collected 48 clinical isolates of *Acinetobacter* spp. from blood cultures, and *A. soli* was the most frequent isolate (27.1%), followed by *A. nosocomialis* (25.0%), *A. baumannii* (18.8%), and *A. ursingii* (16.7%).[30] Moreover, six of the 13 *A. soli* isolates were not susceptible to carbapenem.[30] Therefore, there is an urgent need to provide automated systems to accurately identify *Acinetobacter* species for prevalence tracking and for clinical treatment of patients. Additionally, the dissemination of carbapenem-resistant *Acinetobacter* isolates that are non-*A. baumannii* and non-*A. nosocomialis* should be closely monitored.

Our results showed that the seven AB218-derived transconjugants that carried MBL-plasmids showed higher virulence to larvae compared to the recipient AB218 (Fig. 4D). However, MBL-carrying plasmids did not contribute to the virulence of recipient AN254 (Fig. 4D). These results suggest the presence of virulence genes or regulators in MBL-carrying plasmids which may modulate bacterial virulence to larvae. However, the different expression levels of these genes located in the MBL-carrying plasmids in different species may cause this phenotypic difference.

In recent years, numerous studies have reported the spread of highly virulent and drug-resistant bacteria, often associated with the horizontal transfer of virulence plasmids,[31–33] leading to high patient mortality rates. Rakovitsky et al. isolated nine strains of carbapenem-resistant *A. baumannii* with positive string tests.[34] However, these string test-positive strains generally do not exhibit stronger biofilm formation or higher virulence than string test-negative strains.[34] Therefore, in the future, there should be further research to understand the characteristics and monitor the spread of *Acinetobacter* isolates with high drug resistance and virulence.

The limitations of this study include the fact that all isolates were gathered from a single teaching hospital, making it challenging to compare geographical variations in CNSA. Additionally, the relatively small sample size resulted from the absence of a biobank establishment earlier. However, while CNSA characterization has been documented globally, many studies enrolled isolates for shorter durations. In contrast, our study's strength lies in the 19-year collection, allowing us to report on the longitudinal evolution and epidemiological trends of invasive CNSA isolates.

Conclusions

Our report showed a steady increase in CNSA from 1997 to 2015 and a high rate of *A. soli* among *Acinetobacter* spp. causing bacteremia. In addition, bla_{IMP} and bla_{VIM} are the major MBLs contributing to the carbapenem non-susceptibility of *Acinetobacter*. Our findings emphasize the importance of conducting longitudinal and epidemiological investigations to determine the carbapenem resistance mechanisms of *Acinetobacter*.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The complete genome sequences of *Acinetobacter* have been deposited in GenBank under the accession numbers CP119218-CP119222 (AS366), CP119223-CP119226 (AP54), CP119227-CP119231 (AS435), CP119247-CP119254 (AS566), CP119236-CP119242 (AS645), CP119243-CP119246 (AB839), CP119202-CP119207 (AS843), CP119232-CP119235 (AB322), CP119208-CP119213 (AS903-1), CP119214-CP119217 (AS967-1), CP119255-CP119257 (AJ1044), and CP119200-CP119201 (AJ572). The other data will be made available on request.

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Authors' contributions

JJW contributed to the collection of isolates. PKC, YTL, CYL, TTDT, CHL, JJW, YTH, and KA carried out the experiments and interpreted the results of bacterial identification, antibiotic susceptibility tests, genotypic detection of carbapenemases, and efflux pump inhibitor assay. CYK conceived the study and was in charge of overall direction and planning. CYK was responsible for the manuscript preparation. All authors read and approved the final manuscript. PKC, YTL, and CYL contributed equally to this article (co-first authors).

Declaration of competing interest

The authors report that there are no competing interests to declare.

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References

1. Peleg AY, Seifert H, Paterson DL. Acinetobacter baumannii: emergence of a successful pathogen. *Clin Microbiol Rev* 2008; **21**(3):538—82.
2. Mohd Sazlly Lim S, Zainal Abidin A, Liew SM, Roberts JA, Sime FB. The global prevalence of multidrug-resistance among Acinetobacter baumannii causing hospital-acquired and ventilator-associated pneumonia and its associated mortality: a systematic review and meta-analysis. *J Infect* 2019;**79**(6):593—600.
3. Shin B, Park W. Antibiotic resistance of pathogenic Acinetobacter species and emerging combination therapy. *J Microbiol* 2017;**55**(11):837—49.
4. Barry AL, Jones RN, Thornsberry C, Ayers LW, Kundargi R. Imipenem (N-formimidoyl thienamycin): in vitro antimicrobial activity and beta-lactamase stability. *Diagn Microbiol Infect Dis* 1985;**3**(2):93—104.
5. Rodriguez CH, Nastro M, Famiglietti A. Carbapenemases in Acinetobacter baumannii. Review of their dissemination in Latin America. *Rev Argent Microbiol* 2018;**50**(3):327—33.
6. Codjoe FS, Donkor ES. Carbapenem resistance: a review. *Med Sci (Basel)* 2017;**6**(1).
7. Catel-Ferreira M, Coadou G, Molle V, Mugnier P, Nordmann P, Siroy A, et al. Structure-function relationships of CarO, the carbapenem resistance-associated outer membrane protein of Acinetobacter baumannii. *J Antimicrob Chemother* 2011;**66**(9): 2053—6.
8. Xu C, Bilya SR, Xu W. adeABC efflux gene in Acinetobacter baumannii. *New Microbes New Infect* 2019;**30**:100549.
9. Gupta N, Angadi K, Jadhav S. Molecular characterization of carbapenem-resistant acinetobacter baumannii with special reference to carbapenemases: a systematic review. *Infect Drug Resist* 2022;**15**:7631—50.
10. The Clinical & Laboratory Standards Institute (CLSI). *Performance standards for antimicrobial susceptibility testing*. 30th Edition. Wayne: CLSI supplement M100-S30; 2020. CLSI, 2020.
11. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012;**18**(3):268—81.
12. Chen TL, Lee YT, Kuo SC, Yang SP, Fung CP, Lee SD. Rapid identification of Acinetobacter baumannii, Acinetobacter nosocomialis and Acinetobacter pittii with a multiplex PCR assay. *J Med Microbiol* 2014;**63**(Pt 9):1154—9.
13. Edwards U, Rogall T, Blocker H, Emde M, Bottger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* 1989;**17**(19):7843—53.
14. Lee YT, Fung CP, Wang FD, Chen CP, Chen TL, Cho WL. Outbreak of imipenem-resistant Acinetobacter calcoaceticus-Acinetobacter baumannii complex harboring different carbapenemase gene-associated genetic structures in an intensive care unit. *J Microbiol Immunol Infect* 2012;**45**(1):43—51.
15. Kao CY, Udval U, Huang YT, Wu HM, Huang AH, Bolormaa E, et al. Molecular characterization of extended-spectrum beta-lactamase-producing Escherichia coli and Klebsiella spp. isolates in Mongolia. *J Microbiol Immunol Infect* 2016;**49**(5):692—700.
16. Wick RR, Holt KE. *Benchmarking of long-read assemblers for prokaryote whole genome sequencing*, **8**. F1000Res; 2019. p. 2138.
17. Thuy TTD, Lu HF, Kuo PY, Lin WH, Lin TP, Lee YT, et al. Whole-genome-sequence-based characterization of an NDM-5-producing uropathogenic Escherichia coli EC1390. *BMC Microbiol* 2022;**22**(1):150.
18. Shin SH, Lim Y, Lee SE, Yang NW, Rhee JH. CAS agar diffusion assay for the measurement of siderophores in biological fluids. *J Microbiol Methods* 2001;**44**(1):89—95.
19. Gebhardt MJ, Gallagher LA, Jacobson RK, Usacheva EA, Peterson LR, Zurawski DV, et al. Joint transcriptional control of virulence and resistance to antibiotic and environmental stress in acinetobacter baumannii. *mBio* 2015;**6**(6):e01660. -01615.
20. Tao Y, Duma L, Rossez Y. Galleria mellonella as a good model to study acinetobacter baumannii pathogenesis. *Pathogens* 2021; **10**(11).
21. Ardebili A, Talebi M, Azimi L, Rastegar Lari A. Effect of efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone on the minimum inhibitory concentration of ciprofloxacin in acinetobacter baumannii clinical isolates. *Jundishapur J Microbiol* 2014;**7**(1):e8691.
22. Shields RK, Paterson DL, Tamma PD. Navigating available treatment options for carbapenem-resistant acinetobacter baumannii-calcoaceticus complex infections. *Clin Infect Dis* 2023;**76**(Supplement_2):S179—93.
23. Lin YC, Sheng WH, Chen YC, Chang SC, Hsia KC, Li SY. Differences in carbapenem resistance genes among Acinetobacterbaumannii, Acinetobacter genospecies 3 and Acinetobacter genospecies 13TU in Taiwan. *Int J Antimicrob Agents* 2010;**35**(5): 439—43.
24. Labrador-Herrera G, Perez-Pulido AJ, Alvarez-Marin R, Casimiro-Soriguer CS, Cebrero-Cangueiro T, Moran-Barrio J, et al. Virulence role of the outer membrane protein CarO in carbapenem-resistant Acinetobacter baumannii. *Virulence* 2020;**11**(1):1727—37.
25. Lee Y, Kim CK, Lee H, Jeong SH, Yong D, Lee K. A novel insertion sequence, ISAba10, inserted into ISAba1 adjacent to the bla(OXA-23) gene and disrupting the outer membrane protein gene carO in Acinetobacter baumannii. *Antimicrob Agents Chemother* 2011;**55**(1):361—3.
26. Clark RB. Imipenem resistance among Acinetobacter baumannii: association with reduced expression of a 33-36 kDa outer membrane protein. *J Antimicrob Chemother* 1996;**38**(2): 245—51.
27. Uppalapati SR, Sett A, Pathania R. The outer membrane proteins OmpA, CarO, and OprD of acinetobacter baumannii confer a two-pronged defense in facilitating its success as a potent human pathogen. *Front Microbiol* 2020;**11**:589234.
28. Zhu W, Wang H, Zhang JP. A comparison of adeB gene expression levels under conditions of induced resistance by different drugs in vitro in Acinetobacter baumannii. *Exp Ther Med* 2017;**13**(5):2177—82.
29. Gholami M, Hashemi A, Hakemi-Vala M, Goudarzi H, Hallajzadeh M. Efflux pump inhibitor phenylalanine-arginine beta-naphthylamide effect on the minimum inhibitory concentration of imipenem in acinetobacter baumannii strains isolated from hospitalized patients in shahid motahari burn hospital, tehran, Iran. *Jundishapur J Microbiol* 2015;**8**(10): e19048.
30. Endo S, Yano H, Kanamori H, Inomata S, Aoyagi T, Hatta M, et al. High frequency of Acinetobacter soli among Acinetobacter isolates causing bacteremia at a tertiary hospital in Japan. *J Clin Microbiol* 2014;**52**(3):911—5.

31. Darmancier H, Domingues CPF, Rebelo JS, Amaro A, Dionisio F, Pothier J, et al. Are virulence and antibiotic resistance genes linked? A comprehensive analysis of bacterial chromosomes and plasmids. *Antibiotics (Basel)* 2022;**11**(6).
32. Foley SL, Kaldhone PR, Ricke SC, Han J. Incompatibility group I1 (IncI1) plasmids: their genetics, biology, and public health relevance. *Microbiol Mol Biol Rev* 2021;**85**(2).
33. Guo CY, Guo MQ, Wang YT, Wang SS, Lv C, Li M, et al. Genomic epidemiology of hypervirulent carbapenem-resistant Klebsiella pneumoniae at Jinshan local hospital, Shanghai, during 2014-2018. *J Microbiol Immunol Infect* 2024;**57**(1):128—37.
34. Rakovitsky N, Lurie-Weinberger MN, Hameir A, Wulffhart L, Keren Paz A, Schwartz D, et al. Phenotypic and genomic characterization of nine string-positive carbapenem-resistant acinetobacter baumannii isolates from Israel. *Microbiol Spectr* 2023;**11**(2):e0300222.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.01.008.