ORIGINAL RESEARCH

Comparative analysis of the pathogenicity between multidrug-resistant *Acinetobacter baumannii* clinical isolates: isolation of highly pathogenic multidrug-resistant *A. baumannii* and experimental therapeutics with fourth-generation cephalosporin cefozopran

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Introduction: The pathogenicity of fatal-outbreak *Acinetobacter baumannii* isolates has not been fully investigated. This study aimed to compare the pathogenicity between *A. baumannii* clinical isolates, including multidrug-resistant *A. baumannii* (MDRA).

Materials and methods: Antibiotic susceptibility was determined by the broth microdilution method, and drug-resistant genes were characterized by PCR and sequencing. The pathogenicity of *A. baumannii* and antibiotic responses were evaluated using the *Galleria mellonella* infection model. Clinical isolates from an *A. baumannii* outbreak at our hospital were categorized using the pulse-field gel electrophoresis. Of the 16 isolated *A. baumannii* clones, 12 clones were resistant to carbapenems (meropenem and imipenem), of which 10 clones were also resistant to amikacin and ciprofloxacin (MDRAs). MDRAs had OXA-51-like β-lactamase gene harboring an insertion sequence in the promoter region and *armA* gene encoding 16S rRNA methyltransferase.

Results: Carbapenem- and/or amikacin-resistant *A. baumannii* were more pathogenic than carbapenem- and/or amikacin-sensitive *A. baumannii* in *G. mellonella*. MDRA isolate TK1033 was more virulent than other *A. baumannii* isolates. However, TK1033 was sensitive to the fourth-generation cephalosporin cefozopran in addition to minocycline, tigecycline, and polymyxins (colistin and polymyxins B) in vitro and in vivo in the MDRA-*G. mellonella* infection model.

Conclusion: Differences in pathogenicity among carbapenem-resistant *A. baumannii* clones are consistent with heterogeneous clinical outcomes. Strain TK1033, isolated frequently during the outbreak, was the most virulent, whereas preoutbreak isolate TK1032 was less virulent than other *A. baumannii* isolates. Infection by high-virulence isolates may be more prevalent during outbreaks. These strains may prove valuable for investigating MDRA virulence and novel therapeutics.

Keywords: *Acinetobacter baumannii*, amikacin, aminoglycoside, carbapenem, cephalosporin, cefozopran, virulence, *Galleria mellonella*, multidrug resistance

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Introduction

The *Enterococcus feacium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp. (ESKAPE) pathogens frequently acquire drug resistance. Nosocomial infections from multidrug-resistant (MDR) pathogens often have poor outcomes, especially in intensive care units.

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A. baumannii is a Gram-negative pathogen of opportunistic infection, and MDR *A. baumannii* (MDRA) strains are often resistant to major antibiotics such as amikacin (AMK), ciprofloxacin (CIP), and imipenem (IPM).[1,2]

A fatal MDRA outbreak occurred in 2010 at Teikyo University Hospital (Tokyo, Japan).[3,4] In this study, we compared the pathogenicity of *A. baumannii* clinical isolates including MDRA strains. Despite many clinical studies, little is known regarding the virulence and microbial fitness of *A. baumannii*. The greater wax moth *Galleria mellonella* may be useful for the prospective evaluation of microbial pathogens in randomized controlled studies[5] as infectivity and antibiotic responses show strong concordance with mammalian infection models,[6] particularly for *A. baumannii*.[5,7] Furthermore, this in vivo system has obvious ethical and logistical advantages over mammalian infection models. The objectives of this study were to assess the virulence and antibiotic response profiles of carbapenem-resistant *A. baumannii* (CRAB) isolates from this fatal outbreak using the *G. mellonella* host model.

Materials and methods
Ethics
The Teikyo University Animal Ethics Committee exempted this study.

Materials
Ampicillin (AMP) and kanamycin (KAN) were purchased from Meiji Seika Pharma (Tokyo, Japan). Cefepime (FEP) was purchased from Bristol-Myers Squibb (New York, NY, USA). Colistin (CST), minocycline (MIN), polymyxin B (PMB), and tetracycline (TET) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). AMK and tigecycline (TGC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sitafloxacin (STX) was purchased from Cimic CMO (Tokyo, Japan). Cefozopran (ZOP), doxycycline (DOX), and IPM were generously provided by Takeda Pharmaceutical (Osaka, Japan), Pfizer (New York, NY, USA), and Banyu Pharmaceutical (Tokyo, Japan), respectively. CIP, sparfloxacin (SPX), and meropenem (MEM) were generously provided by Sumitomo Dainippon Pharma (Osaka, Japan). Lysogeny broth (LB; Miller), LB ager (Miller), and Muller Hinton Broth II (MHB II) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Cation-adjusted MHB II was prepared by adding $CaCl_2$ and $MgCl_2$ (Wako Pure Chemical Industries) at the final concentrations of 50 mg/L Ca^{2+} and 25 mg/L Mg^{2+} to autoclaved MHB II. Saline was prepared as 0.9% NaCl (Wako Pure Chemical Industries).

Isolation and characterization of *A. baumannii*
A. baumannii clinical isolates were previously collected from Teikyo University Hospital, a 1,082-bed academic hospital in Tokyo, Japan,[4] and stored in glycerol at −80°C. During the MDRA outbreak from 2009 to 2010, 59 patients were infected and nine patients who had hematological diseases died as a result of the infection. *A. baumannii* strains were originally isolated from blood, sputum, stool, urine, bronchoalveolar lavage, pus, and pharyngeal mucus specimens. All clinical isolates of *A. baumannii* were identified at the species level by the *rpoB* sequence.[8] Carbapenem resistance was confirmed by the broth microdilution method. Antibiotic susceptibility was interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (document M100-S22). Each isolate exhibited a unique pattern on pulse-field gel electrophoresis (PFGE) (Kawakami, in preparation, 2018). Frozen stocks of *A. baumannii* were streaked on LB agar and incubated at 37°C. *A. baumannii* isolates were grown at 37°C in LB for liquid culture in Bioshaker BR-40LF (Taitec, Tokyo, Japan).

Gene amplification and DNA sequencing
A genomic fragment was amplified by PCR using KOD FX Neo (Toyobo, Osaka, Japan). RNA polymerase β-subunit gene *rpoB* was amplified according to a previously described protocol.[8] DNA sequences were determined by direct sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Sequences were compared using NCBI BLASTN 2.7.1. Presence of the bla_{OXA-51}-like gene was confirmed by PCR with primers OXA-51A (5′-CTAATAATTGATCTACTCAAG-3′) and OXA-51B (5′-CCAGTGGATGGATGGATAGATTATC-3′), while presence of the *armA* gene was determined using primes armA-F (5′-AGGTTGTTTCCATTTCTGAG-3′) and armA-R (5′-ATCTCTTCCATTCCCTTCTCC-3′).[9,10]

Antimicrobial activity
The minimum inhibitory concentrations (MICs, in milligram per liter) were determined by the broth microdilution technique according to CLSI guidelines (document M7-A9). The MIC was defined as the lowest antimicrobial concentration of a serial twofold dilution (0.25–256 mg/L) in cation-adjusted MHB II conferring no growth (<2 mm button or no definite turbidity) of *A. baumannii* on 96-well plates (BMBio, Tokyo, Japan).

Infection model

G. mellonella larvae (250–350 mg) were purchased from Live Bait Factory (Osaka, Japan). Each isolate was diluted in saline (0.9% NaCl) and 10 µL was injected into the rear right proleg of 10 randomly selected *G. mellonella* larvae using an insulin syringe (Terumo, Tokyo, Japan). Saline (0.9% NaCl) and injection-free groups (both n=10) were used to evaluate the effects of trauma and attrition, respectively. *G. mellonella* larvae were incubated at 37°C. Death was defined as unresponsiveness to touch.[7] Survival curves were plotted using Kaleidagraph 4.1.4 (Synergy Software, Reading, PA, USA), and median lethal dose (LD_{50}) values were determined by curve fitting at 24h.[11]

For evaluating antibacterial activity against MDRA, 10 µL of antimicrobial agents were injected into the rear left proleg of 10 *G. mellonella* larva infected with MDRA TK1033. Saline was used as a control of agents. Antimicrobial doses were 40 mg/kg each.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2007 for Windows (Redmond, WA, USA) and Excel Statistics 2008 (Social Survey Research Information, Tokyo, Japan). Survival plots were generated by the Kaplan-Meier method and compared among antibiotics by the log-rank test. Differences between log CFU values were tested for significance by one-way ANOVA followed by Fisher's least significant difference tests for pair-wise comparisons. A *P*-value of <0.05 was considered to be statistically significant. All tests were two-tailed.

Results

Pathogenicity of *A. baumannii* clinical isolates in *G. mellonella*

Sixteen unique pulse-field gel electrophoresis (PFGE) isolates were tested in the *G. mellonella* infection model. Colony-forming units were used to confirm the injected inocula. Injection of *A. baumannii* isolates resulted in time-dependent and dose-dependent larval death, but LD_{50} values varied markedly (Table 1). We then compared the LD_{50} values among drug-sensitive, MEM/CIP-resistant, CIP/AMK-resistant, and MEM/CIP/AMK-resistant (MDRA) isolates (Figure 1). The LD_{50} values of drug-sensitive isolates were comparable to double-resistant isolates MEM/CIP (*P*=0.59) and CIP/AMK (*P*=0.13). In contrast, the LD_{50} values of MDR isolates (MEM/CIP/AMK) were significantly lower (higher virulence) than those of drug-sensitive isolates (*P*=0.010) and double-resistant (MEM/CIP) isolates (*P*=0.0027). The LD_{50} values of CIP/

AMK-resistant isolates were significantly lower (higher virulence) than those of MEM/CIP-resistant isolates (*P*=0.046). However, the LD_{50} values of MEM/CIP/AMK-resistant isolates were comparable to double-resistant (CIP/AMK) isolates (*P*=0.21). These data suggest that AMK resistance is a critical determinant of *A. baumannii* virulence. Similarly, the LD_{50} values of the 10 MDRA isolates were comparable to

Table 1 Summary of *Acinetobacter baumannii* clinical isolates

Strain	Source	CIP	AMK	MEM	LD_{50} (CFU)
ATCC 19606	ATCC[a]	S	S	S	4.9×10^5
TK1020	This study[b]	R	R	R	4.6×10^4
TK1021	This study[b]	R	R	S	2.4×10^4
TK1022	This study[b]	R	R	R	1.9×10^5
TK1023	This study[b]	S	S	S	2.1×10^5
TK1024	This study[b]	R	S	R	1.7×10^5
TK1025	This study[b]	R	R	R	9.7×10^3
TK1026	This study[b]	R	R	S	5.9×10^4
TK1027	This study[b]	R	R	R	1.6×10^4
TK1028	This study[b]	R	R	S	4.6×10^4
TK1029	This study[b]	R	R	R	4.8×10^3
TK1030	This study[b]	R	R	R	4.3×10^4
TK1031	This study[b]	R	R	R	1.4×10^4
TK1032	This study[b]	R	S	R	2.9×10^6
TK1033	This study[b]	R	R	R	8.4×10^2
TK1034	This study[b]	R	R	R	4.4×10^3
TK1035	This study[b]	R	R	R	3.6×10^3

Notes: R, AMK, CIP, and MEM MICs ≥32, 4, and 16 mg/L, respectively. [a]Isolated from urine. [b]Isolated from blood, sputum, stool, urine, bronchoalveolar lavage, pus, and pharyngeal mucus specimens.
Abbreviations: AMK, amikacin; ATCC, American Type Culture Collection; CFU, colony-forming units; CIP, ciprofloxacin; LD_{50}, median lethal dose; MEM, meropenem; MICs, minimum inhibitory concentrations; R, resistant, S, sensitive.
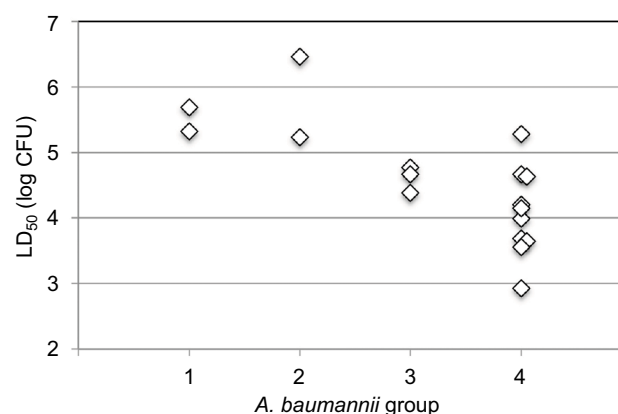

Figure 1 LD_{50} values of different *A. baumannii* strains in *G. mellonella*.
Notes: The LD_{50} values expressed in log CFU are plotted for four *A. baumannii* groups: (1) drug sensitive, (2) MEM/CIP resistant, (3) CIP/AMK resistant, and (4) MEM/CIP/AMK resistant (MDR). MDR isolates (MDRA) induced significantly greater mortality than the drug-sensitive group and the MEM/CIP-resistant isolates group (*P*=0.010 and 0.0027, respectively).
Abbreviations: *A. baumannii*, *Acinetobacter baumannii*; AMK, amikacin; CIP, ciprofloxacin; *G. mellonella*, *Galleria mellonella*; LD_{50}, median lethal dose; MEM, meropenem; MDR, multidrug resistant; MDRA, MDR *A. baumannii*.
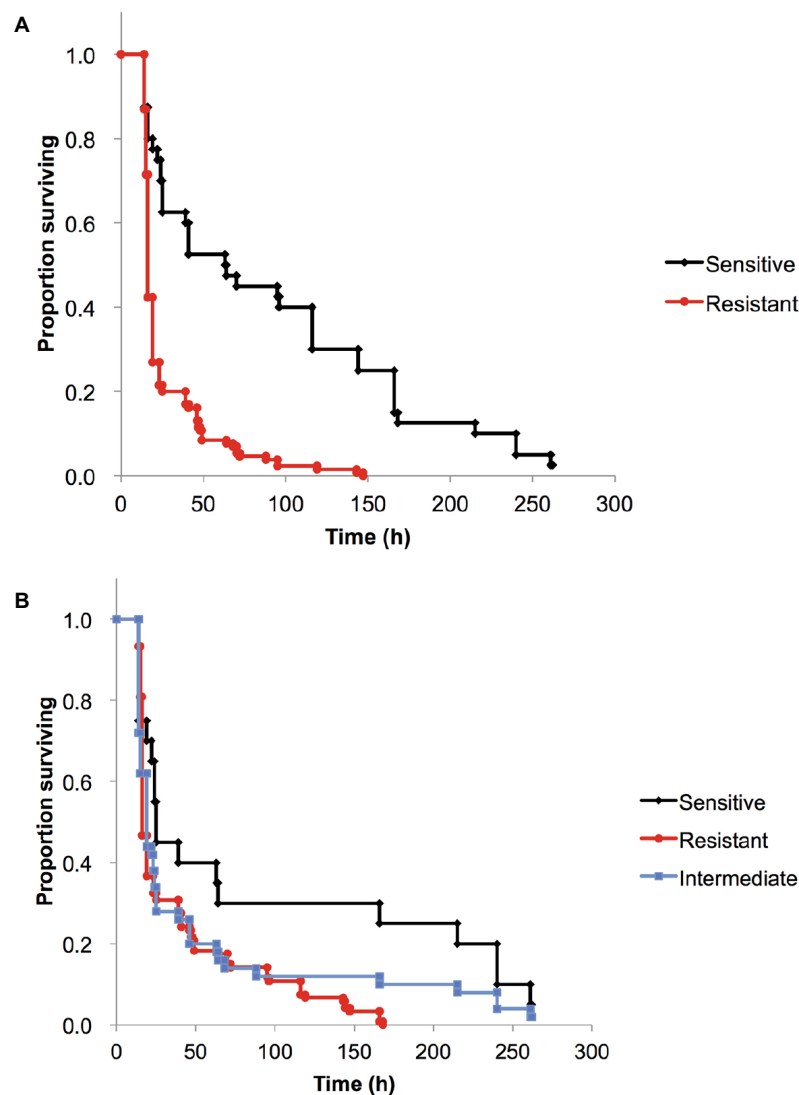
Figure 2 Kaplan–Meier cumulative survival curves of *G. mellonella* infected with *A. baumannii*.
Notes: *G. mellonella* larvae were infected with 1×10⁵ CFU of each strain. Survival curves for the three drug-resistant groups were compared using the log-rank test. (**A**) AMK-resistant *A. baumannii* (MIC ≥32 mg/L) induced significantly greater mortality than AMK-sensitive *A. baumannii* ($P=2.5×10^{-7}$). (**B**) CRAB (MEM MIC ≥32 mg/L) induced significantly greater mortality than CSAB (MEM MIC ≤2 mg/L) ($P=0.011$) and comparable mortality than *A. baumannii* of intermediate carbapenem resistance (MEM MIC 4–16 mg/L) ($P=0.47$).
Abbreviations: AMK, amikacin; *A. baumannii*, *Acinetobacter baumannii*; CFU, colony-forming units; CRAB, carbapenem-resistant *A. baumannii*; CSAB, carbapenem-sensitive *A. baumannii*; *G. mellonella*, *Galleria mellonella*; MEM, meropenem; MIC, minimum inhibitory concentration.

those of the four double-resistant CIP/aminoglycoside (AMK or KAN) isolates ($P=0.10$).

Next, we compared the survival of *G. mellonella* infected with AMK-sensitive and -resistant isolates. At 24 h postinoculation, the LD_{50} values of the 13 AMK-resistant isolates were lower than those of the four AMK-sensitive isolates (Figure 2A). Furthermore, *G. mellonella* larvae were killed more rapidly by the 13 AMK-resistant isolates than the four AMK-sensitive isolates ($P=2.5×10^{-7}$). For instance, after 2 days, 62% of the *G. mellonella* larvae inoculated with AMK-sensitive isolates survived, compared to only 20% inoculated with

AMK-resistant isolates (Figure 2A). The presence of the *armA* gene coding 16S rRNA methyltransferase was confirmed in all AMK-resistant isolates by PCR, suggesting that *armA*-positive isolates are generally more virulent against *G. mellonella* larvae than *armA*-negative isolates (except for the AMK-sensitive, KAN-resistant and *armA*-positive isolate TK1024).

CRAB isolates also induced greater mortality of *G. mellonella* than carbapenem-sensitive *A. baumannii* (CSAB) isolates ($P=0.011$, Figure 2B). The isolates with intermediate carbapenem resistance showed virulence comparable to CRAB ($P=0.47$) and CSAB ($P=0.13$). In our study, *G. mellonella* lar-

vae were killed more rapidly by bla_{OXA-23}-negative isolates of *A. baumannii* than bla_{OXA-23}-positive isolate TK1032 (Table 1). These results suggest that clinical isolates harboring the bla_{OXA-23} gene and those involved in an outbreak may be less virulent.

Antibiotic sensitivity of highly virulent MDRA

Next, we evaluated the antibiotic sensitivity of the MDRA isolate TK1033 (Table 2). TK1033 was resistant to aminoglycosides such as KAN and AMK. TK1033 was also resistant to the TET antibiotics TET and DOX but sensitive to the TET antibiotics MIN and TGC. Similarly, TK1033 was sensitive to the fourth-generation quinolone antibiotic STX but resistant to the second-generation quinolone CIP and the third-generation quinolone SPX. TK1033 was also sensitive to the polymyxins PMB and CST. TK1033 was resistant to the fourth-generation cephalosporin FEP but sensitive to another fourth-generation cephalosporin, ZOP.

Antibacterial treatment of MDRA with the fourth-generation cephalosporin ZOP

It has previously been shown that appropriate antimicrobials can successfully treat *G. mellonella* infected with *A. baumannii*.[7] To determine whether the *G. mellonella*-MDRA infection model can be used to study the in vivo activity of antibacterial agents against our clinical isolates, we investigated the effects of single-dose (40 mg/kg) ZOP, CST, PMB, MIN, TGC, and STX on MDRA-infected *G. mellonella* larvae (Figure 3). Initially, we infected *G. mellonella* larvae with the MDRA strain TK1033. Treatment with antibiotics that had in vitro activity against this *A. baumannii* strain significantly prolonged the survival of *G. mellonella* larvae. Thus, the *G. mellonella*-MDRA infection model can be used as an alternative to mammalian systems for the assessment of antibacterial agent efficacy.

Discussion

The pathogenicity of *A. baumannii* clinical strains has been studied using *G. mellonella*. The US military clinical isolate

AB5075 exhibited a 100-fold lower LD_{50} in the *G. mellonella* infection model than strain American Type Culture Collection (ATCC) 19606.[12] Another clinical strain, ATCC 17978, exhibited a twofold lower LD_{50} than ATCC 19606. In contrast, the epidemiological outbreak strains AYE and ACICU presented about twofold higher LD_{50} values than ATCC 17978.[13] Therefore, *A. baumannii* strains show a broad range of virulence values and resistance profiles in vitro that do not always correspond with clinical outcome. Thus, the clinical relevance of different strain characteristics in epidemiological outbreaks is uncertain. In this study, we selected clinical isolates previously classified at our institution to analyze the relevance of individual resistance spectra to clinical virulence. MDR (MEM/CIP/AMK) isolates of *A. baumannii* appeared to be more virulent than double-resistant (MEM/CIP and CIP/AMK) isolates (Figure 1). In particular, TK1033 exhibited a 500-fold lower LD_{50} in the *G. mellonella* infection model than strain ATCC 19606 (Table 1). Isolates with the same PFGE pattern as the highly virulent strain TK1033 were most frequently isolated during the outbreak, suggesting clinically significant infectivity and pathogenicity to humans. However, extrapolation of these results to human virulence will require additional study using a mouse infection model. Our previous report has suggested that the lipopolysaccharide (LPS) subtype of the outbreak strain is also a possible virulence factor. Indeed, the LPS from TK1033 induced greater expression of proinflammatory cytokine genes in human polymorphonuclear leukocytes than that from a drug-sensitive *A. baumannii* in vitro.[14] Some studies have reported hypervirulent strains in epidemic outbreak isolates and have analyzed the pathogenicity in animal infection models.[12,15–17] The horizontal clonal spread of hypervirulent strains may contribute to epidemic outbreaks and poor clinical outcomes. Further study is warranted to elucidate virulence in animal models and clinical outcomes.

Recently, Lemos et al (2014)[18] conducted a systematic review and meta-analysis of carbapenem resistance and mortality in patients with *A. baumannii* infection. CRAB-infected patients showed significantly higher mortality risk than CSAB-infected patients, although there was a substantial

Table 2 MIC values (mg/L) of the *Acinetobacter baumannii* clinical isolates in this study

Strain	AMP	IPM	MEM	FEP	ZOP	CIP	SPX	STX	KAN	AMK	TET	DOX	MIN	TGC	PMB	CST
ATCC 19606	128	0.25	1	32	16	1	0.25	0.25	8	8	8	0.125	1	2	0.5	2
TK1033	>256	8	32	16	8	64	16	4	>256	>256	>256	16	8	8	1	4

Abbreviations: AMK, amikacin; AMP, ampicillin; CIP, ciprofloxacin; CST, colistin; DOX, doxycycline; FEP, cefepime; IPM, imipenem; KAN, kanamycin; MEM, meropenem; MIC, minimum inhibitory concentration; MIN, minocycline; PMB, polymyxin B; SPX, sparfloxacin; STX, sitafloxacin; TET, tetracycline; TGC, tigecycline; ZOP, cefozopran.
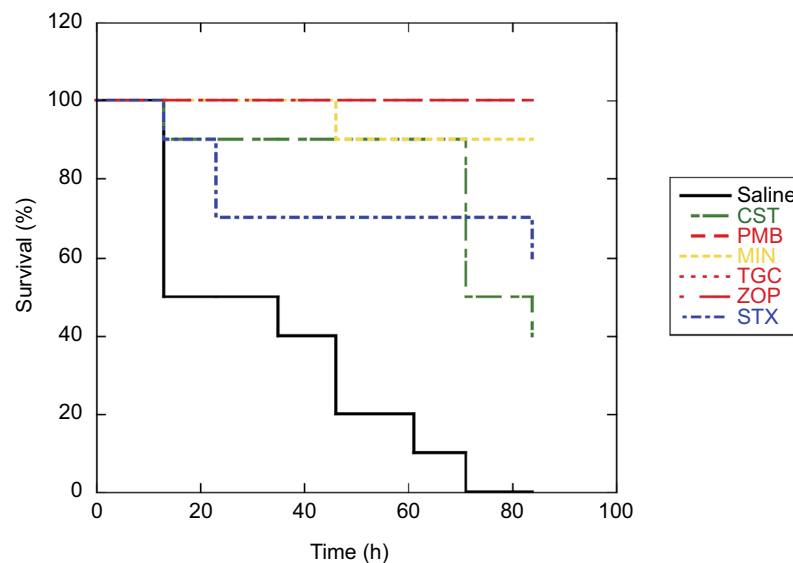
Figure 3 Antibacterial activities against MDRA TK1033.
Notes: After infection with MDRA TK1033 (5×10^3 CFU), ZOP, STX, MIN, TGC, PMB, and CST (40 mg/kg each), antibiotics to which the strain was susceptible, significantly prolonged the survival of infected *G. mellonella* larvae ($P<0.01$ compared to saline-treated control infected larvae).
Abbreviations: CFU, colony-forming units; CST, colistin; *G. mellonella*, *Galleria mellonella*; MDRA, multidrug-resistant *Acinetobacter baumannii*; MIN, minocycline; PMB, polymyxin B; STX, sitafloxacin; TGC, tigecycline; ZOP, cefozopran.

variation in outcome. A case-control study of mortality following CRAB bacteraemia suggested a clonal subgroup of *A. baumannii* as a predictor of mortality.[19] Therefore, differences in virulence among CRAB clones may partly contribute to the heterogeneous results in clinical studies. The results of the present study appear consistent with those of previous clinical studies, suggesting that carriage of carbapenem-resistance genes is associated with organism virulence, although with some variance (Figure 2B). While these studies demonstrated increased mortality among patients with CRAB vs CSAB bloodstream infections on bivariate inspection, this does not exclude the possibility that patient comorbidities drive poor outcomes or that patients with more comorbidities are more likely to be infected by CRAB.

In our study, *G. mellonella* larvae were killed more rapidly by bla_{OXA-23}-negative isolates of *A. baumannii* than bla_{OXA-23}-positive isolate of *A. baumannii*. These results suggest that clinical isolates harboring the bla_{OXA-23} gene and those involved in an outbreak may be less virulent, consistent with a previous study regarding clinical isolates harboring the bla_{OXA-40} gene and those involved in an outbreak.[5] However, another study reported a highly virulent isolate, AB5075, harboring bla_{OXA-23} and involved in an outbreak.[12] Hence, the impact of carbapenem resistance on mortality due to *A. baumannii* infection remains uncertain.

In contrast to bla_{OXA-23}-positive isolate, AMK-resistant isolates of *A. baumannii* killed *G. mellonella* larvae more rapidly than AMK-sensitive isolates (Figure 2A). These results suggest that clinical isolates harboring the *armA* gene coding 16S rRNA methyltransferase and those involved in an outbreak may be more virulent. The impact of AMK resistance and/or the *armA* gene on mortality due to *A. baumannii* infection warrants further study in light of this possibility.

In this study, we utilized *G. mellonella* to compare the pathogenicity of *A. baumannii* clinical isolates and to assess the efficacy of antibiotic treatment against a highly pathogenic MDRA. Very little is known about the pathogenicity of MDRA, so this infection model may serve as a useful tool for future research. Interestingly, we observed an association between the pathogenicity of MDRA in *G. mellonella* and that observed in humans. Therefore, this model may also facilitate the study of novel compounds against MDRA with the ethical and logistical advantages over mammalian models. Mammalian infection models should be used to confirm our results before human studies in the future.

The third-generation cephalosporins are widely used for the empiric therapy of serious nosocomial infections. However, expression of β-lactamase (eg, AmpC) may confer resistance. Fourth-generation cephalosporins have potent

activity against Gram-negative bacteria including those producing AmpC β-lactamase.[20,21] ZOP is a fourth-generation cephalosporin approved for various bacterial infections in Japan.[22] The highly virulent MDRA TK1033 isolate was more sensitive to ZOP than FEP (Table 2). TK1033 was also sensitive to MIN and TGC, both of which are used for empiric therapy of MDRA infection.[23–27] Intravenous MIN is particularly promising due to its improved safety profile.[25–27] ZOP and/or MIN were used for empiric therapy during the MDRA outbreak in our hospital. Antibacterial activities of ZOP and MIN in vitro and in vivo were confirmed in this study (Table 2 and Figure 3). Re-evaluation and use of fourth-generation cephalosporins and MIN may help reduce the use of carbapenems, resulting in reduced carbapenem-resistance for antimicrobial stewardship.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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