J Antimicrob Chemother 2021; **76**: 1205–1214
doi:10.1093/jac/dkab018 Advance Access publication 3 February 2021

Journal of Antimicrobial Chemotherapy

Molecular characteristics and *in vitro* effects of antimicrobial combinations on planktonic and biofilm forms of *Elizabethkingia anophelis*

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Received 29 May 2020; accepted 6 January 2021

Objectives: To investigate the *in vitro* activity of antibiotics against clinical *Elizabethkingia anophelis* isolates and to find a suitable antibiotic combination with synergistic effects to combat antibiotic-resistant *E. anophelis* and its associated biofilm.

Methods: *E. anophelis* isolates were identified by 16S rRNA sequencing; 30 strains with different pulsotypes were identified and the MIC, antibiotic resistance mechanism, antibiotic combination activity and killing effects of antimicrobial agents on biofilms of these strains were determined.

Results: All *E. anophelis* isolates were susceptible to minocycline and cefoperazone/sulbactam (1:1). More than 90% of clinical isolates were susceptible to cefoperazone/sulbactam (1:0.5), piperacillin/tazobactam and rifampicin. Some novel mutations, such as *gyrA* G81D, *parE* D585N and *parC* P134T, that have never been reported before, were identified. The synergistic effect was most prominent for the combination of minocycline and rifampicin, with 93.3% of their FIC index values $\leq$0.5, and no antagonism was observed using the chequerboard method. This synergistic effect between minocycline and rifampicin was also observed using time–killing methods for clinical *E. anophelis* isolates at both normal inoculum and high inoculum. Twenty-nine isolates tested positive for biofilm formation. Minocycline remained active against biofilm-embedded and biofilm-released planktonic *E. anophelis* cells; however, the enhanced effect of minocycline by adding rifampicin was only observed at 24 h (not at 72 and 120 h).

Conclusions: Although *E. anophelis* was resistant to many antibiotics and could exhibit biofilm formation, minocycline showed potent *in vitro* activity against this pathogen and its associated biofilm.

Introduction

The genus *Elizabethkingia* (Gram-negative, glucose-non-fermenting bacilli) is ubiquitously distributed in natural environments, including water and soil, as well as hospital tap water.[1,2] After it was first identified in 1959, human infections caused by *Elizabethkingia* species have been reported in clinical settings, as healthcare-associated infections.[1] Thereafter, the incidence of infections associated with *Elizabethkingia* increased rapidly.[1,2] *Elizabethkingia meningoseptica* and *Elizabethkingia anophelis* are two major pathogens within the genus *Elizabethkingia*. However, the incidence of *E. anophelis* infection may be underestimated due to its frequent misidentification as *E. meningoseptica* in previous studies using conventional laboratory methods.[2] *E. anophelis* infections present as bacteraemia, pneumonia, catheter-related bloodstream infections, meningitis, skin and soft-tissue infections, urinary tract infections and biliary tract infections;[2–4] in addition,

several risk factors have been associated with *E. anophelis* infection, including age over 60 years old, diabetes mellitus, malignancy, chronic kidney disease, liver cirrhosis, alcoholism, immunocompromised status and receiving immunosuppressive treatment.[3–9] In addition, several outbreaks of *E. anophelis* infections have been reported in Singapore[10] and the USA.[5,6] Most importantly, the mortality rate of outbreaks of *E. anophelis* infections has been reported to be up to 24%–60%.[5,6,10]

Appropriate antibiotics are the key to treating infections and saving lives; however, *E. anophelis* is notorious for its high resistance to many antibiotics, including β-lactams, carbapenems, aminoglycosides and β-lactam/β-lactamase inhibitors.[3,4,11,12] In addition, the susceptibility rates of *E. anophelis* to piperacillin, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, ciprofloxacin and levofloxacin are variable.[2] Although the resistance rate of *E. anophelis* to minocycline remains low, novel agents or additional antibiotic combinations to enhance our antibiotic armamentarium are needed. Recently, two novel β-lactam/β-lactamase inhibitors, ceftazidime/avibactam and ceftolozane/tazobactam, were developed and approved for clinical use.[13–15] However, their activity against *E. anophelis* remains unknown. Therefore, this study was conducted to investigate the *in vitro* activity of antibiotics, including two novel β-lactam/β-lactamase inhibitors, ceftazidime/avibactam and ceftolozane/tazobactam, and a traditional cefoperazone/sulbactam, against clinical *E. anophelis* isolates. In addition, the antibiotic resistance mechanisms among these *E. anophelis* isolates were also evaluated. This study also aimed to find a suitable antibiotic combination with synergistic effects to combat antibiotic-resistant *E. anophelis*.

Methods

Bacterial isolates

Sixty-three *E. anophelis* clinical isolates were collected from the Taipei Veterans General Hospital, the National Cheng Kung University Hospital and the Chi-Mei Medical Center between 2014 and 2019. Species were initially identified using the Vitek MS system with the IVD 3.0 database (bioMérieux, Marcy-l'Étoile, France).[3] Species were confirmed by 16S rRNA sequencing using the following primers: 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492 R (5′-GGYTACCTTGTTAC GACTT-3′). After PCR amplification, the amplicons were separated by gel electrophoresis and sequenced. Sequences were compared with the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST) to find the closest matches.[16] The isolates were stored at −80°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, UK) before use.[17]

PFGE analysis

The *E. anophelis* isolates were characterized by PFGE using a CHEF DR II apparatus (Bio-Rad Laboratories, Hercules, CA, USA) with the restriction endonuclease XbaI as described previously.[18] Briefly, bacterial chromosomal DNAs were digested using XbaI (New England Biolabs, Beverly, MA, USA). Electrophoresis was carried out for 22 h at 14 μC, with pulse times ranging from 2 to 40 s at 6 V/cm, using a Bio-Rad CHEF MAPPER apparatus (Bio-Rad Laboratories, Richmond, CA, USA). The PFGE patterns were visually examined and interpreted according to the criteria of Tenover *et al.*[19] The Dice similarity coefficients were calculated and PFGE profiles with <80% similarity were considered different pulsotypes. Thirty different pulsotypes were selected for further studies (Figure 1).

Antibiotics and MIC determination

The antibiotics tested were: ceftazidime, ceftriaxone, cefepime, amikacin, gentamicin, doripenem, imipenem, meropenem, minocycline, tigecycline, ciprofloxacin, levofloxacin, colistin, rifampicin, trimethoprim/sulfamethoxazole, piperacillin, tazobactam, ticarcillin and clavulanic acid (Sigma, St Louis, MO USA); cefoperazone and sulbactam (United States Pharmacopeia, Rockville, MD, USA); avibactam (MedKoo Biosciences, Inc., Morrisville, NC, USA); and ceftolozane/tazobactam (bioMérieux, Marcy-l'Étoile, France). Except for ceftolozane/tazobactam, all antibiotic MICs were determined by the agar dilution method and interpretation criteria and were based on the recommendations of the CLSI.[20] Briefly, Mueller–Hinton agar (Oxoid, Basingstoke, UK) was employed to determine the MICs for *E. anophelis*. Inocula were prepared by suspending overnight cultures in saline to a turbidity equivalent to that of a 0.5 McFarland standard. Inoculated plates were then incubated in ambient air at 37°C for 24 h. The MIC of ceftolozane/tazobactam was determined by Etest according to the manufacturer's recommendations. Inocula were prepared by directly suspending overnight cultures in Mueller–Hinton broth to achieve a turbidity equivalent to that of a 0.5 McFarland opacity standard. The 10 cm diameter agar plates were inoculated by confluent swabbing of the surface with the adjusted inoculum suspensions. Etest strips were placed on the surface of the plates and incubated at 35°C for 16–18 h. Quality control testing was performed using *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853.[21]

PCR detection and sequencing of antibiotic resistance genes

PCR using specific primers (Table S1, available as Supplementary data at *JAC* Online) was used to amplify the ESBL (CME) and MBL (GOB and BlaB) genes and to screen for mutations in the QRDRs of *gyrA, gyrB, parC* and *parE* in quinolone-resistant strains.[4,22] Amplicons of β-lactamase genes were purified with PCR clean-up kits (Roche Diagnostics GmbH, Penzberg, Germany) and sequenced on an ABI PRISM 3730 sequencer analyser (Applied Biosystems, Foster City, CA, USA). Sequences were analysed using BLAST online through the NCBI database,[23] ResFinder and CARD databases.

Phenotypic methods for the detection of ESBLs

Combination discs, including cefotaxime/clavulanate and ceftazidime/clavulanate (BBL Sensi-Disc, Becton Dickinson, Cockeysville, MD, USA), were used to detect ESBLs. A ≥5 mm increase in zone diameter for cefotaxime or ceftazidime in combination with clavulanate compared with the zone when tested alone was indicative of an ESBL phenotype. Control experiments were performed by testing *E. coli* ATCC 25922 and *K. pneumoniae* 700603.[24]

Antibiotic combinations assessed by the chequerboard method

To evaluate the effect of the combinations, the microdilution chequerboard method was used to calculate the FICs, as recommended by the CLSI.[20,25] Briefly, 96-well flat-bottom microtitre plates (Nunc, Thermo Scientific, Denmark) were inoculated with each test organism to yield the appropriate density (1×10^5 cfu/mL) in 100 μL of Mueller–Hinton broth and incubated at 35°C in ambient air for 24 h. The plates were read for visual turbidity and the MIC, which was the lowest drug concentration at which there was no visible growth, was determined from the microtitre plates as well. The following formula was used to calculate the FIC index: FIC of drug A (MIC of drug A in combination/MIC of drug A alone)+FIC of drug B (MIC of drug B in combination/MIC of drug B alone). Synergism was defined as an FIC index ≤0.5, indifference was defined as an FIC index >0.5–4 and antagonism was defined as an FIC index >4.[25] Repeats for all drug combinations were performed to validate the data.

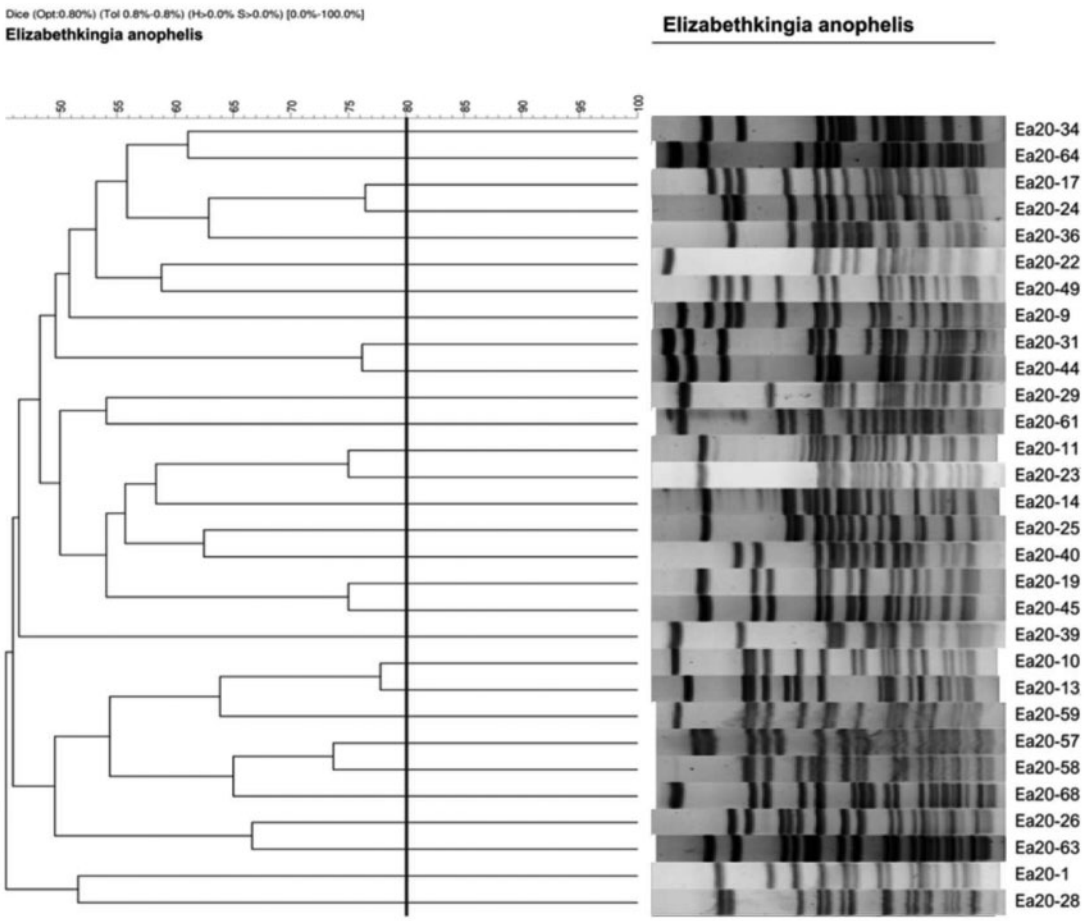

Figure 1. PFGE patterns of 30 *E. anophelis* isolates. The number on the scale is the percentage of genetic similarity. PFGE profiles with <80% similarity were considered different.

Antibiotic combinations assessed by the broth method

The *in vitro* inhibitory effect of combination regimens following the broth killing method was defined in accordance with the CLSI (formerly the NCCLS).[26] In brief, 15 randomly selected strains were tested and the bacterial suspensions were diluted to concentrations of 5×10^5 (normal inoculum) and 5×10^7 (high inoculum) cfu/mL in fresh Mueller–Hinton broth. Drug concentrations of minocycline and rifampicin were adjusted to 0.5×MIC for the normal inoculum and to the susceptible breakpoint concentration (SBC) for the high inoculum. SBC was defined as the SBC for other non-Enterobacteriaceae of minocycline and *Staphylococcus* of rifampicin. Each drug was tested alone and in combination. Bacterial counts were measured at 0, 4, 24, 48 and 72 h; colonies were serially diluted 10-fold in 100 μL aliquots, plated on nutrient agar (Difco Laboratories, Sparks, MD, USA) at 37°C and enumerated.

Biofilm formation

The biofilm formation test was modified from our previous study.[27] In brief, all 30 strains were cultured for 1 day at 37°C in 5 mL of tryptic soy broth (Difco Laboratories) supplemented with 1% D-glucose (TSBGlc). The cultures were diluted 1:1000 in TSBGlc and 200 μL of the final solution was added to each well of a 96-well tissue culture-treated polystyrene plate. After 24 h of growth at 37°C, the plates were washed vigorously three times with PBS to remove unattached bacteria and stained with 1% crystal violet. The plate was incubated for 15 min at 25°C; the staining solution was removed and the plate was washed three times with PBS. After removing the washing solution, 100 μL of DMSO was added to each well to dissolve the biofilm-bound crystal violet and incubated for 5 min. The OD was obtained as an index of adherent bacteria and biofilm formation. To compensate for background absorbance, the OD of a sterile medium with fixative and dye was recorded and subtracted from the results. All strains were classified into the following categories (where ODc is the mean OD of the negative control): non-biofilm formation, OD <ODc; weak biofilm formation, OD >ODc–2×ODc; moderate biofilm formation, OD >2×ODc–4×ODc; and strong biofilm formation, OD >4×ODc.[28] The experiments were performed in triplicate and the results presented as mean ± SD.

Minimum biofilm eradication concentration (MBEC)

The MBEC assay was performed using a 96-well polystyrene flat-bottom plate as previously described.[29] Briefly, the biofilms were formed on plastic pegs on the lid of the MBEC plate (Nunc, Thermo Scientific, Denmark). These biofilms were exposed to antibiotics for 24 h at 37°C, placed in a second 96-well plate containing fresh Mueller–Hinton broth and incubated overnight. The MBEC was the lowest dilution that prevented bacterial regrowth after antibiotic treatment.

Killing effects of antimicrobial agents on biofilms

The biofilms of each isolate were prepared in 24-well culture plates. The medium in the wells was removed by aspiration and the biofilm in each

plate was treated with minocycline (4 mg/L) or rifampicin (1 mg/L) at the SBC or minocycline in combination with rifampicin. The antibiotic-containing medium was gently aspirated after 24 h and the biofilm was washed with PBS three times. Fresh antibiotic-containing medium was added to the wells continuously for 5 days.

To quantify the degree of inhibition of biofilm-released (planktonic) and biofilm-embedded bacteria by the tested antibiotics, the cell suspension and biofilm were collected on days 0 (before antibiotic treatment), 1, 3 and 5. The planktonic bacteria were detected using the broth method described above. The wells containing biofilms were sonicated using a water-table sonicator for 5 min. The disrupted biofilm was serially diluted, plated and cultured overnight at 37°C and viable cells were counted. The limitation of detection in this study was 2 $\log_{10}$ cfu/mL. All tests were performed in triplicate.[27]

Statistical analysis

Data analyses were performed using SPSS for Windows 17.0 (SPSS Inc., Chicago, IL, USA). The Mann–Whitney *U*-test was used to compare the differences between the two groups. The Kruskal–Wallis *H*-test and Dunn's test were applied for multiple comparisons. The statistical significance was set at a P value <0.05.

Results

MIC results

Table 1 shows the MIC results of 23 tested antibiotics for 30 *E. anophelis* isolates. All *E. anophelis* isolates were susceptible to minocycline and cefoperazone/sulbactam (1:1). More than 90% of clinical isolates were susceptible to cefoperazone/sulbactam (1:0.5), piperacillin/tazobactam and rifampicin, and 83.3% were susceptible to piperacillin. In addition, 73.3% and 70.0% of isolates were susceptible to trimethoprim/sulfamethoxazole and levofloxacin, respectively. For the other β-lactam/β-lactamase inhibitor combinations, the susceptibility rates of ceftolozane/tazobactam, ticarcillin/clavulanic acid and ceftazidime/avibactam were only 16.7%, 3.3% and 0%, respectively. Finally, all isolates were resistant to gentamicin, ceftazidime, ceftriaxone, cefepime, doripenem, imipenem, meropenem, tigecycline and colistin.

Carbapenem and quinolone resistance and associated antibiotic resistance mechanism

Table 2 shows the MICs of carbapenems and quinolones for *E. anophelis* isolates and the associated resistance genes. The MICs of the three carbapenems for all isolates were high and ESBL phenotypes were observed for all isolates. All of the *E. anophelis* isolates carried resistance genes for the MBLs GOB and BlaB and the ESBL CME, except one isolate (Ea20-61) that lacked BlaB. Regarding quinolone resistance, three mutations were found in the *gyrA* gene (S83I, S83R and G81D) and only one mutation was found in the *parC* (P134T) and *parE* genes (D585N). Any mutation in the *gyrA* and *parE* genes resulted in quinolone resistance. Only the mutation in *parC* did not result in quinolone resistance (conservative missense mutation).

Table 1. MIC results of 23 tested antibiotics for 30 *E. anophelis* isolates

	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Susceptible (%)
Amikacin	16 to >128	64	>128	3.3
Gentamicin	16 to >128	128	>128	0
Ceftazidime	>128	>128	>128	0
Ceftriaxone	>128	>128	>128	0
Cefepime	>128	>128	>128	0
Doripenem	32 to >128	64	128	0
Imipenem	32–128	64	128	0
Meropenem	32 to >128	64	128	0
Minocycline	0.25–2	0.5	1	100
Tigecycline	4–128	16	32	0
Ciprofloxacin	0.5 to >128	2	>128	26.7
Levofloxacin	0.25–64	1	64	70.0
Colistin	>128	>128	>128	0
Rifampicin	0.25 to >128	0.5	2	90
Trimethoprim/sulfamethoxazole	2/38 to >32/608	2/38	16/304	73.3
Piperacillin	8–32	16	32	83.3
Piperacillin/tazobactam	8/4–32/4	16/4	16/4	93.3
Cefoperazone	16–64	32	64	6.7
Cefoperazone/sulbactam (1:0.5)	8–32	8	16	96.7
Cefoperazone/sulbactam (1:1)	4–16	4	8	100
Ceftolozane/tazobactam[a,b]	2/4–48/4	3/4	6/4	16.7
Ceftazidime/avibactam	>128/4	>128/4	>128/4	0
Ticarcillin/clavulanic acid	4/2 to >128/2	>128/2	>128/2	3.3

[a]Breakpoints are based on a dosage regimen of 1.5 g every 8 h.
[b]Determined by Etest.

Table 2. MICs of carbapenems and quinolones for *E. anophelis* isolates and the associated resistance genes

	ESBL		MBL										
	CME gene	phenotype	GOB gene	BlaB gene	Doripenem MIC (mg/L)	Meropenem MIC (mg/L)	Imipenem MIC (mg/L)	*gyrA*	*gyrB*	*parC*	*parE*	Levofloxacin MIC (mg/L)	Ciprofloxacin MIC (mg/L)
Ea20-1	+	+	+	+	64	64	128	WT	WT	WT	WT	0.5	2
Ea20-9	+	+	+	+	32	32	32	WT	WT	WT	WT	1	4
Ea20-10	+	+	+	+	128	128	128	WT	WT	WT	WT	2	8
Ea20-11	+	+	+	+	32	32	64	WT	WT	WT	WT	1	2
Ea20-13	+	+	+	+	64	128	64	WT	WT	WT	WT	1	2
Ea20-14	+	+	+	+	32	32	64	WT	WT	WT	WT	0.5	1
Ea20-17	+	+	+	+	64	64	64	WT	WT	WT	WT	0.5	1
Ea20-19	+	+	+	+	64	128	64	WT	WT	WT	WT	1	2
Ea20-22	+	+	+	+	64	128	128	WT	WT	WT	WT	1	0.5
Ea20-23	+	+	+	+	32	32	64	WT	WT	WT	WT	1	2
Ea20-24	+	+	+	+	64	64	64	WT	WT	WT	WT	1	2
Ea20-25	+	+	+	+	64	128	64	WT	WT	**P134T**	WT	1	1
Ea20-26	+	+	+	+	64	64	64	**S83I**	WT	WT	**D585N**	64	>128
Ea20-28	+	+	+	+	64	64	64	WT	WT	WT	WT	0.5	2
Ea20-29	+	+	+	+	64	64	64	WT	WT	WT	WT	0.5	1
Ea20-31	+	+	+	+	128	>128	128	**S83I**	WT	WT	WT	32	>128
Ea20-34	+	+	+	+	64	64	64	WT	WT	WT	WT	0.25	0.5
Ea20-36	+	+	+	+	64	64	64	WT	WT	WT	WT	1	2
Ea20-39	+	+	+	+	128	128	128	WT	WT	WT	WT	2	32
Ea20-40	+	+	+	+	64	64	64	WT	WT	WT	WT	0.5	1
Ea20-44	+	+	+	+	64	64	64	WT	WT	WT	WT	0.5	2
Ea20-45	+	+	+	+	64	64	64	WT	WT	WT	WT	1	2
Ea20-49	+	+	+	+	64	64	64	WT	WT	WT	WT	0.5	1
Ea20-57	+	+	+	+	>128	>128	128	**S83I**	WT	WT	**D585N**	64	>128
Ea20-58	+	+	+	+	64	64	64	**S83R**	WT	WT	**D585N**	16	128
Ea20-59	+	+	+	+	128	128	128	**G81D**	WT	WT	WT	64	32
Ea20-61	+	+	+	−	128	64	128	**S83R**	WT	WT	WT	16	128
Ea20-63	+	+	+	+	128	64	64	**S83I**	WT	WT	**D585N**	32	>128
Ea20-64	+	+	+	+	64	64	64	**S83R**	WT	WT	WT	32	>128
Ea20-68	+	+	+	+	64	64	64	**S83R**	WT	WT	**D585N**	16	>128

ESBL phenotype was determined by combination disc test.

Activity of antibiotics in combination

The activity of seven antibiotic combinations was assessed by the chequerboard method and is shown in Table 3. The synergistic effect was most prominent for the combination of minocycline and rifampicin, with 93.3% of their FIC index values $\leq$0.5, and no antagonism was found. In contrast, antagonism (FIC index >4) was found in two-thirds of isolates with the combination of minocycline and piperacillin/tazobactam. Excluding the combination of minocycline and rifampicin, the other three rifampicin-based combinations exhibited indifference against at least 60% of isolates and no obvious synergistic effect was found.

Further time–killing tests revealed that with 0.5×MIC of rifampicin, there was no obvious inhibitory effect. With 0.5×MIC of minocycline, there was a mild inhibitory effect at 4 h and then regrowth (Figure 2a). However, compared with rifampicin or minocycline alone, the combination of minocycline and rifampicin significantly reduced the number of colonies, which persisted between 24 and 72 h (Figure 2a). With the high inoculum and rifampicin at the SBC, there was no obvious inhibitory effect. With minocycline at the SBC, there was a mild inhibitory effect until 48 h and then regrowth. However, the combination of minocycline and rifampicin significantly reduced the number of colonies to 10^4 cfu/mL at 72 h compared with rifampicin ($P<0.001$) or minocycline ($P<0.05$) alone (Figure 2b).

Biofilm

Twenty-nine isolates tested positive for biofilm formation, with OD values $\geq$0.17 at 590 nm (isolate Ea20-9 was the only isolate that did not test positive). Moreover, eight isolates tested positive for strong biofilm formation, with OD values $\geq$1.0, and six of them (Ea20-1, Ea20-17, Ea20-29, Ea20-31, Ea20-39 and Ea20-61) had the highest OD values, with values $\geq$2.0 (Figure 3). The MBECs of levofloxacin, minocycline, rifampicin, piperacillin, piperacillin/tazobactam, cefoperazone/sulbactam (1:1) and trimethoprim/sulfamethoxazole for these six isolates were >128 mg/L, except the

Table 3. Results of seven antibiotic combinations assessed by the chequerboard method according to the following criteria: synergism, FIC index ≤0.5; indifference, FIC index >0.5–4; and antagonism, FIC index >4

	FIC index ≤0.5 (%)	FIC index >0.5–4 (%)	FIC index >4 (%)	FIC index range	$FIC\ index_{50}$	$FIC\ index_{90}$
MIN versus TZP	0	33.3	66.7	1–8.5	4.25	8.5
MIN versus SXT	0	60	40	0.625–8.5	2.25	8.125
MIN versus LVX	0	93.3	6.7	1–4.125	2.5	2.5
MIN versus RIF	93.3	6.7	0	0.375–0.75	0.5	0.5
RIF versus TZP	0	60	40	1–16.5	2.5	4.5
RIF versus SXT	6.7	93.3	0	0.375–1.5	0.75	1.5
RIF versus LVX	6.7	93.3	0	0.254–2.5	1	2.5

MIN, minocycline; TZP, piperacillin/tazobactam; SXT, trimethoprim/sulfamethoxazole; LVX, levofloxacin; RIF, rifampicin.

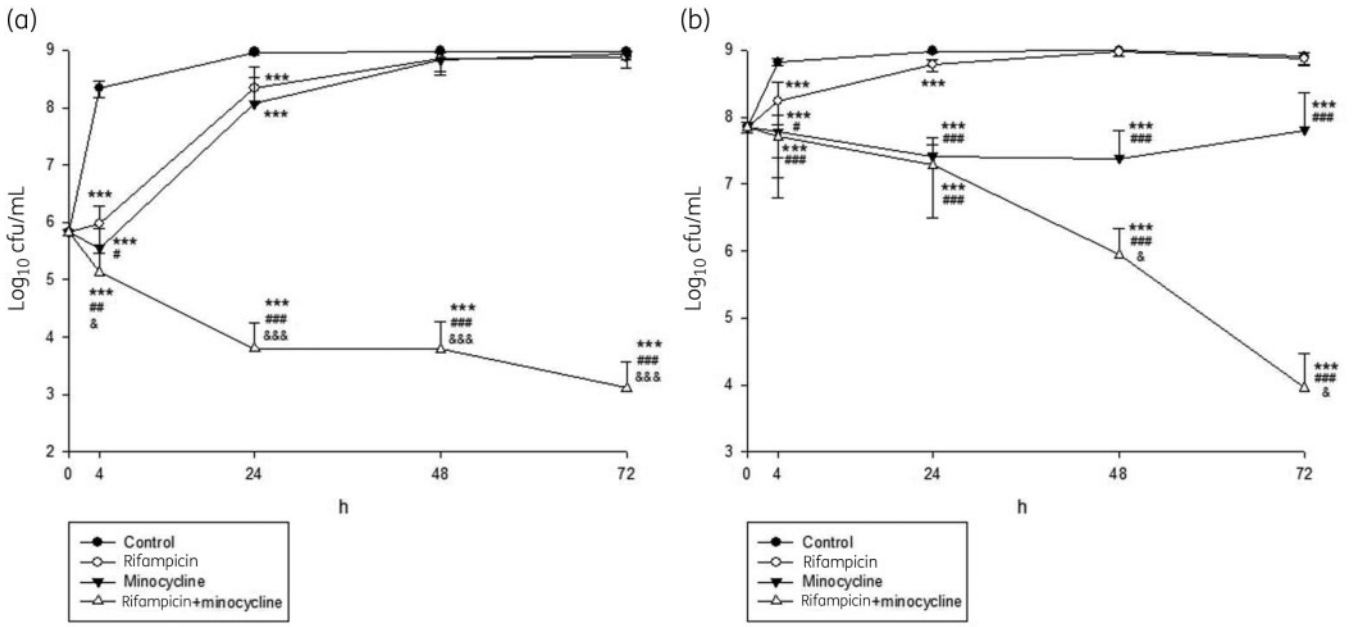

Figure 2. Results of time–killing tests of minocycline, rifampicin and the combination of minocycline and rifampicin against 15 randomly selected *E. anophelis* isolates at normal inoculum (a) and high inoculum (b) with 0.5× of the SBC. *Compared with control. #Compared with rifampicin. &Compared with minocycline. *, # and &: $P < 0.05$. **, ## and &&: $P < 0.01$. ***, ### and &&&: $P < 0.001$.

MBECs of rifampicin for two isolates that were 128 mg/L (Ea20-1) and 64 mg/L (Ea20-31).

Furthermore, the effect of antibiotic combination on the six strongest biofilm-embedded *E. anophelis* was tested using time–killing methods. Although rifampicin alone at the SBC could significantly inhibit growth at 24 h, no more inhibition was found at 48–72 h. In contrast, the inhibitory effect of minocycline alone was also mild at 24 h, but continued killing at 48–72 h. In addition, the effect of minocycline on *E. anophelis* biofilms was enhanced by adding rifampicin at 24 h, but no enhancement by rifampicin could be observed at 72 and 120 h (Figure 4a).

With the same concentrations, we tested the planktonic isolates from the supernatant and found that the colony count of *E. anopheles* was high, with a concentration more than $>10^9$ cfu/mL. The inhibitory effect of the combination at 24 h was significantly enhanced compared with rifampicin alone ($P < 0.001$) and minocycline alone ($P < 0.01$). Moreover, the inhibitory effect of the combination and minocycline alone could persist for 72 and even 120 h (Figure 4b). However, the enhancement of minocycline activity by adding rifampicin was only observed at 24 h. No significant difference was observed between the rifampicin/minocycline combination and minocycline alone against planktonic ($>10^9$ cfu/mL) *E. anophelis* at 72 and 120 h (Figure 4b).

Even when 0.5× of the SBC was used (Figure 5a), the colony count of biofilm-embedded *E. anophelis* decreased to 10^2 and 10^4 cfu/mL at 72 h for the combination and minocycline alone, respectively, and the inhibitory effect persisted until 120 h. For the planktonic *E. anophelis*, the phenomenon was the same at 72 h and the colony count decreased to 10^2 and 10^4 cfu/mL with the combination and minocycline alone, respectively. Moreover, the colony growth could be totally inhibited at 120 h with minocycline alone or in combination with rifampicin (Figure 5b).

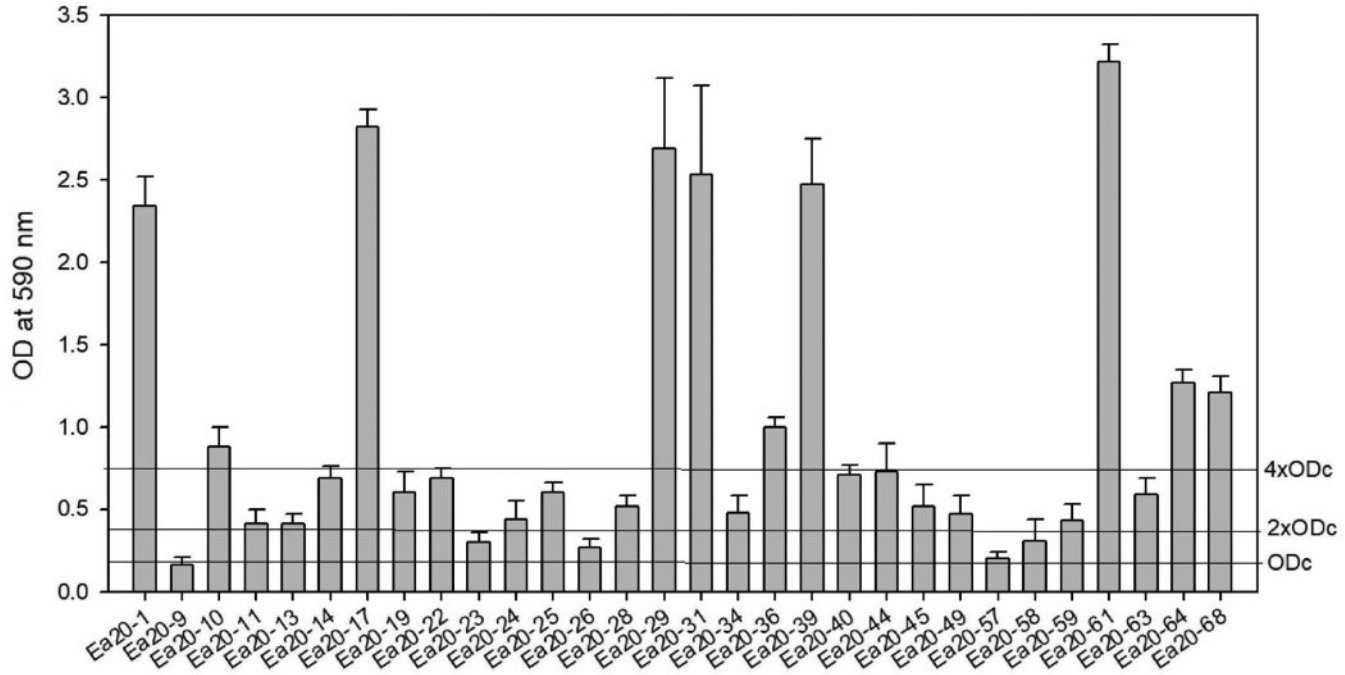

Figure 3. Biofilm formation results of the *E. anophelis* isolates using the following criteria: non-biofilm formation, OD <ODc; weak biofilm formation, OD >ODc–2×ODc; moderate biofilm formation, OD >2×ODc–4×ODc; and strong biofilm formation, OD >4×ODc.

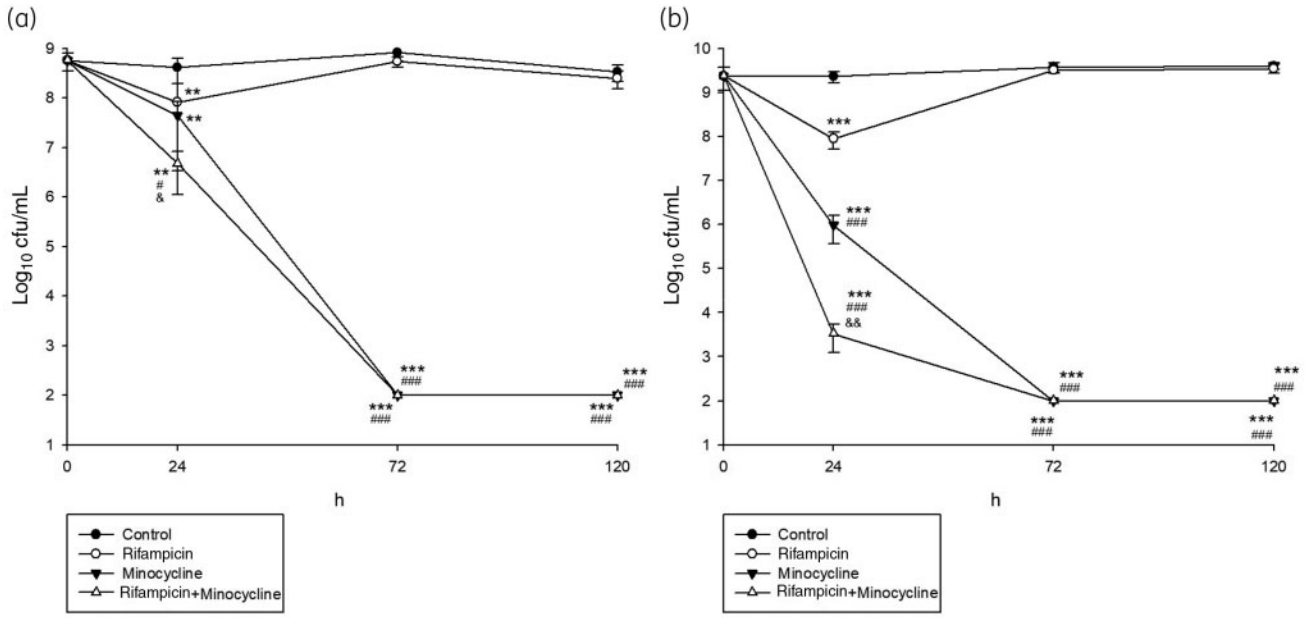

Figure 4. Effect of antibiotic combination on the six biofilm-embedded (about 5.0×10^8 cfu/mL) (a) and planktonic (about 2.4×10^9 cfu/mL) (b) *E. anophelis* with strongest biofilm formation using time–killing methods with SBCs. *Compared with control. #Compared with rifampicin. &Compared with minocycline. *, # and &: $P < 0.05$. **, ## and &&: $P < 0.01$. ***, ### and &&&: $P < 0.001$.

Discussion

This study investigated the microbiological characteristics of *E. anophelis* clinical isolates, which were confirmed by molecular methods to avoid misidentification by conventional methods.[12]

First, we found that the *E. anophelis* isolates exhibited high resistance to most of the test antibiotics, including carbapenems and two novel β-lactam/β-lactamase inhibitor combinations. In this study, all 23 *E. anophelis* isolates were resistant to ceftazidime,

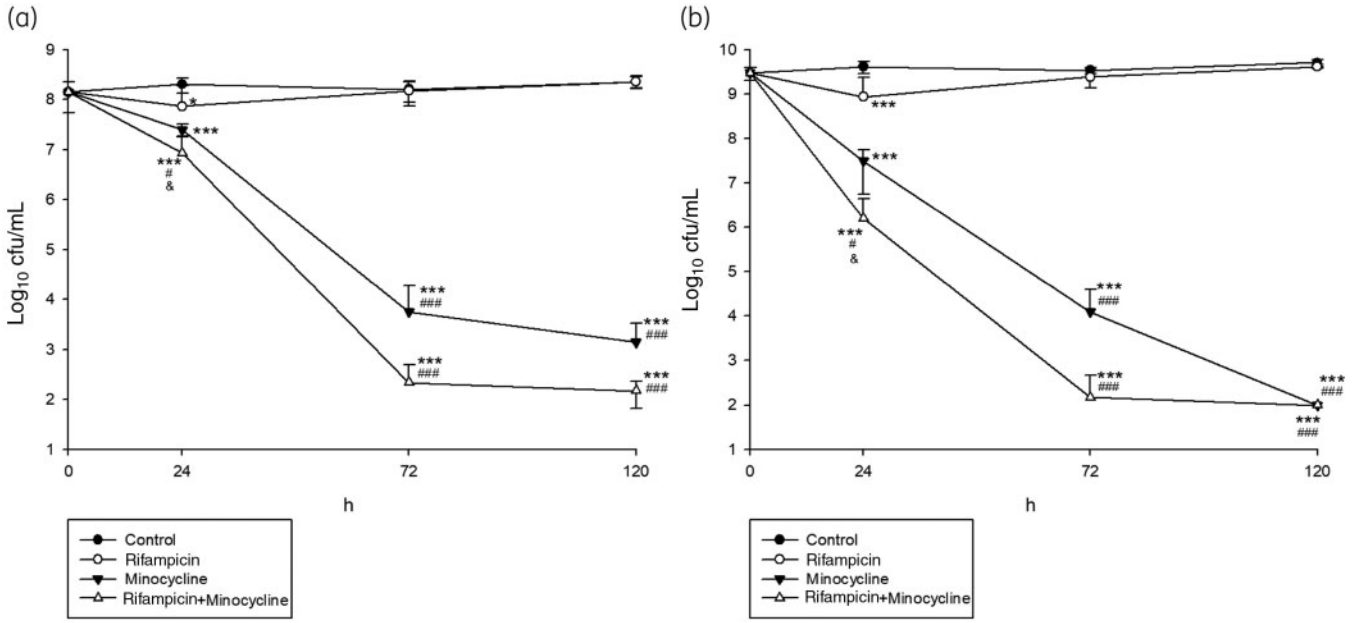

Figure 5. Effect of antibiotic combination on the six biofilm-embedded (about 2.1×10^8 cfu/mL) (a) and planktonic (about 3.0×10^9 cfu/mL) (b) *E. anopheles* with strongest biofilm formation using time–killing methods with 0.5× of the SBC. *Compared with control. #Compared with rifampicin. &Compared with minocycline. *, # and &: $P < 0.05$. **, ## and &&: $P < 0.01$. ***, ### and &&&: $P < 0.001$.

ceftriaxone, cefepime, imipenem, meropenem and colistin (Table 1). This kind of intrinsic resistance of *E. anophelis* was the same as that of *E. meningoseptica*. Regarding carbapenem resistance, we found that almost all *E. anophelis* isolates carried resistance genes for the MBLs GOB and BlaB and the ESBL CME. In contrast, two other conventional β-lactam/β-lactamase inhibitor combinations, cefoperazone/sulbactam and piperacillin/tazobactam, showed potent activity against more than 90% of clinical isolates. Although previous studies[12,30,31] showed similar findings regarding the great potency of piperacillin/tazobactam against *E. anophelis* isolates, this study is the first (to the best of our knowledge) to demonstrate the potent activity of cefoperazone/sulbactam and poor activity of ceftolozane/tazobactam and ceftazidime/avibactam against *E. anophelis*. Regarding quinolones, this study found that levofloxacin exhibited good activity, consistent with previous studies in the USA[31] and Singapore,[30] but ciprofloxacin did not, consistent with other studies in Taiwan.[4,11] Regarding the resistance mechanisms of *E. anophelis*, we found several novel mutations, including the mutations *gyrA* G81D, *parE* D585N and *parC* P134T, that have never been reported before. Among them, *parC* P134T, with a proline to threonine mutation, was a conservative missense mutation that did not lead to an increase in the quinolone MIC. Single or double mutations in *gyrA* and *parE* will lead to an increase in the MIC, especially that of ciprofloxacin, which may have an MIC >128 mg/L. Overall, the MIC of ciprofloxacin was higher than that of levofloxacin, which is compatible with a lower susceptibility rate (26.7% versus 70%). The different susceptibility rates of the two fluoroquinolones still need to be further investigated. Finally, we found that minocycline remained the most active antibiotic against most of the isolates, consistent with previous reports in Taiwan and Singapore.[4,11,29] In addition to minocycline, the MIC_{50} and MIC_{90} of rifampicin were only 0.5 and 2 mg/L, with an overall 90% susceptibility rate, which was consistent with the study of Han *et al*.[12] in South Korea. In summary, although the antibiotic resistance pattern could vary at each study site, most reports, such as this study, suggest that minocycline and piperacillin/tazobactam have great *in vitro* activity against *E. anophelis*. Moreover, our findings indicated that cefoperazone/sulbactam exhibited good activity, but ceftolozane/tazobactam and ceftazidime/avibactam did not. However, further large-scale studies are warranted to confirm these new findings and investigate the mechanism causing these different activities within the same antibiotic class, β-lactam/β-lactamase inhibitor combinations.

Second, this study tried to investigate the activity of several minocycline- and rifampicin-based antibiotic combinations to identify the most active formula using a chequerboard assay. We found that the combination of minocycline and rifampicin exhibited the most synergistic effect on *E. anophelis* isolates. This synergistic effect was further demonstrated with time–killing assays (Figure 2). Additionally, this synergistic effect was observed with a high inoculum of *E. anophelis* (Figure 2). All the above findings suggest that the combination of minocycline and rifampicin may be the appropriate antibiotic combination regimen for *E. anophelis* infections.

Third, biofilm formation of antibiotic-resistant organisms could increase the protection of bacteria from external stresses, such as the host immune system and antimicrobial agents. Further dispersal of microcolonies of cells from the main community via protease activation leads to migration to new surfaces, spreading the infection to other locations, which negatively affects the activity of antibiotic treatment and remains a serious concern in eradicating microorganisms.[32] There is no exception for *E. anophelis*.

Our study simulated real biofilm conditions to test not only the inhibitory effect of biofilm-embedded isolates but also the effect of planktonic isolates released from biofilms. In this study, almost

all *E. anophelis* isolates were found to be biofilm-positive and six of them with strong biofilm formation ability showed high MBECs of seven tested antibiotics. We also found extremely high colony counts in supernatants from *E. anophelis* biofilms. Therefore, we tested the effect of antibiotic combination on the biofilm and the planktonic isolates released from the biofilm using time–killing methods and found that the killing effects of the rifampicin/minocycline combination and minocycline alone on biofilm-embedded *E. anophelis* were much better than those of rifampicin alone, even at 0.5× of the SBC. A significant difference between the rifampicin/minocycline combination and minocycline alone was only observed at 24 h (not at 72 and 120 h). Moreover, despite the large number of planktonic *E. anophelis* released from mature biofilm to the supernatant, minocycline still showed a great inhibitory effect. Although rifampicin can enhance the inhibitory effect of minocycline at 24 h, no significant difference between minocycline alone and the rifampicin/minocycline combination was observed at 72 and 120 h. Based on the findings of this *in vitro* study regarding biofilm, it was suggested that minocycline remained the most potent agent against *E. anopheles* biofilm formation. Adding rifampicin only enhanced the inhibitory effect of minocycline on *E. anopheles* biofilm formation at 24 h.

In conclusion, *E. anophelis* exhibited resistance to many antibiotics, including carbapenem and even novel β-lactam/β-lactamase inhibitors; however, minocycline, cefoperazone/sulbactam and piperacillin/tazobactam showed potent activity against this bacterium. As in previous studies,[4,9,10] we found that the *E. anophelis* isolates could have several resistance mechanisms, including the MBLs GOB and BlaB and the ESBL CME, but we found two new mutations in *gyrA* and *parE*. The combination of minocycline and rifampicin exhibited a significant synergic effect, which was demonstrated using the chequerboard method. Although biofilm formation was observed for almost all tested isolates, minocycline remained active against both biofilm-embedded and planktonic *E. anopheles*.

Funding

This study was supported by a research grant (MOST 109-2314-B-384-008) from the Ministry of Science and Technology.

Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

References

1 Jean SS, Lee WS, Chen FL *et al. Elizabethkingia meningoseptica*: an important emerging pathogen causing healthcare-associated infections. *J Hosp Infect* 2014; **86**: 244–9.

2 Lin JN, Lai CH, Yang CH *et al. Elizabethkingia* infections in humans: from genomics to clinics. *Microorganisms* 2019; **7**: 295.

3 Lau SK, Chow WN, Foo CH *et al. Elizabethkingia anophelis* bacteremia is associated with clinically significant infections and high mortality. *Sci Rep* 2016; **6**: 26045.

4 Lin JN, Lai CH, Yang CH *et al.* Comparison of clinical manifestations, antimicrobial susceptibility patterns, and mutations of fluoroquinolone target genes between *Elizabethkingia meningoseptica* and *Elizabethkingia anophelis* isolated in Taiwan. *J Clin Med* 2018; **7**: 538.

5 *Elizabethkingia*. https://www.dhs.wisconsin.gov/disease/elizabethkingia.htm.

6 CDC. *Elizabethkingia* - Recent Outbreaks. https://www.cdc.gov/elizabethkingia/outbreaks/.

7 Choi MH, Kim M, Jeong SJ *et al.* Risk factors for *Elizabethkingia* acquisition and clinical characteristics of patients, South Korea. *Emerg Infect Dis* 2019; **25**: 42–51.

8 Navon L, Clegg WJ, Morgan J *et al.* Notes from the field: investigation of *Elizabethkingia anophelis* cluster - Illinois, 2014-2016. *MMWR Morb Mortal Wkly Rep* 2016; **65**: 1380–1.

9 Lin JN, Lai CH, Yang CH *et al.* Clinical manifestations, molecular characteristics, antimicrobial susceptibility patterns and contributions of target gene mutation to fluoroquinolone resistance in *Elizabethkingia anophelis*. *J Antimicrob Chemother* 2018; **73**: 2497–502.

10 Teo J, Tan SY, Tay M *et al.* First case of *E anophelis* outbreak in an intensive-care unit. *Lancet* 2013; **382**: 855–6.

11 Cheng YH, Perng CL, Jian MJ *et al.* Multicentre study evaluating matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of clinically isolated *Elizabethkingia* species and analysis of antimicrobial susceptibility. *Clin Microbiol Infect* 2019; **25**: 340–5.

12 Han MS, Kim H, Lee Y *et al.* Relative prevalence and antimicrobial susceptibility of clinical isolates of *Elizabethkingia* species based on 16S rRNA gene sequencing. *J Clin Microbiol* 2017; **55**: 274–80.

13 Lin WT, Lai CC, Cheong CU. Novel β-lactam/β-lactamase combination versus meropenem for treating nosocomial pneumonia. *Antibiotics (Basel)* 2019; **8**: 219.

14 Tan CK, Lai CC, Chao CM. Ceftazidime-avibactam versus meropenem for the treatment of complicated intra-abdominal infections. *Antibiotics (Basel)* 2019; **8**: 255.

15 Cheng IL, Chen YH, Lai CC *et al.* The use of ceftolozane-tazobactam in the treatment of complicated intra-abdominal infections and complicated urinary tract infections-a meta-analysis of randomized controlled trials. *Int J Antimicrob Agents* 2020; **55**: 105858.

16 Jian MJ, Perng CL, Sun JR *et al.* Multicentre MDR *Elizabethkingia anophelis* isolates: novel random amplified polymorphic DNA with capillary electrophoresis systems to rapid molecular typing compared to genomic epidemiology analysis. *Sci Rep* 2019; **9**: 1806.

17 Chang PC, Chen CC, Lu YC *et al.* The impact of inoculum size on the activity of cefoperazone-sulbactam against multidrug resistant organisms. *J Microbiol Immunol Infect* 2018; **51**: 207–13.

18 Weaver KN, Jones RC, Albright R *et al.* Acute emergence of *Elizabethkingia meningoseptica* infection among mechanically ventilated patients in a long-term acute care facility. *Infect Control Hosp Epidemiol* 2010; **31**: 54–8.

19 Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; **33**: 2233–9.

20 CLSI. *Performance Standards for Antimicrobial Susceptibility Testing—Twenty-Eighth Edition: M100*. 2018.

21 Shields RK, Clancy CJ, Pasculle AW *et al.* Verification of ceftazidime-avibactam and ceftolozane-tazobactam susceptibility testing methods against carbapenem-resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa*. *J Clin Microbiol* 2018; **56**: e01093-17.

22 Jian MJ, Cheng YH, Chung HY *et al.* Fluoroquinolone resistance in carbapenem-resistant *Elizabethkingia anophelis*: phenotypic and genotypic characteristics of clinical isolates with topoisomerase mutations and comparative genomic analysis. *J Antimicrob Chemother* 2019; **74**: 1503–10.

23 Lai CC, Chen CC, Lu YC *et al*. Simultaneous three Enterobacteriaceae with different bla_{NDM-1}-encoding plasmids in a patient transferred from mainland China to Taiwan. *Infect Drug Resist* 2018; **11**: 2555–60.

24 Ku YH, Chen CC, Lee MF *et al*. Comparison of synergism between colistin, fosfomycin and tigecycline against extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates or with carbapenem resistance. *J Microbiol Immunol Infect* 2017; **50**: 931–9.

25 Moody JA. Synergy testing: broth microdilution checkerboard and broth macrodilution methods. In: Eisenberg HD, ed. *Clinical Microbiology Procedures Handbook*. American Society for Microbiology, 1992; 5.18.1–23.

26 NCCLS. *Methods for Determining Bactericidal Activity of Antimicrobial Agents: Approved Guideline M26-A*. 1999.

27 Tang HJ, Chen CC, Cheng KC *et al*. *In vitro* efficacy of fosfomycin-containing regimens against methicillin-resistant *Staphylococcus aureus* in biofilms. *J Antimicrob Chemother* 2012; **67**: 944–50.

28 Stepanovic S, Vukovic D, Dakic I *et al*. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods* 2000; **40**: 175–9.

29 Ceri H, Olson ME, Stremick C *et al*. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol* 1999; **37**: 1771–6.

30 Chew KL, Cheng B, Lin RTP *et al*. *Elizabethkingia anophelis* is the dominant *Elizabethkingia* species found in blood cultures in Singapore. *J Clin Microbiol* 2018; **56**: e01445-17.

31 Perrin A, Larsonneur E, Nicholson AC *et al*. Evolutionary dynamics and genomic features of the *Elizabethkingia anophelis* 2015 to 2016 Wisconsin outbreak strain. *Nat Commun* 2017; **8**: 15483.

32 de la Fuente-Nunez C, Reffuveille F, Fernandez L *et al*. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Curr Opin Microbiol* 2013; **16**: 580–9.