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Comparative Genomics and Antimicrobial Resistance Profiling of *Elizabethkingia* Isolates Reveal Nosocomial Transmission and *In Vitro* Susceptibility to Fluoroquinolones, Tetracyclines, and Trimethoprim-Sulfamethoxazole

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ABSTRACT The *Elizabethkingia* genus has gained global attention in recent years as containing sporadic, worldwide, nosocomial pathogens. *Elizabethkingia* spp. are intrinsically multidrug resistant, primarily infect immunocompromised individuals, and are associated with high mortality (~20 to 40%). As yet, gaps remain in our understanding of transmission, global strain relatedness, antimicrobial resistance, and effective therapy. Over a 16-year period, 22 clinical and 6 hospital environmental isolates were collected from Queensland, Australia. Identification using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Vitek MS) and whole-genome sequencing was compared with a global strain data set. Phylogenomic reconstruction robustly identified 22 *Elizabethkingia anophelis*, 3 *Elizabethkingia miricola*, 2 *Elizabethkingia meningoseptica*, and 1 *Elizabethkingia bruuniana* isolates, most of which branched as unique lineages. Global analysis revealed that some Australian *E. anophelis* isolates are genetically closely related to strains from the United States, England, and Asia. Comparative genomics of clinical and environmental strains identified evidence of nosocomial transmission in patients, indicating probable infection from a hospital reservoir. Furthermore, broth microdilution against 39 antimicrobials revealed almost ubiquitous resistance to aminoglycosides, carbapenems, cephalosporins, and penicillins. Like other international strains, our isolates expressed susceptibility to minocycline and levofloxacin and the less common trimethoprim-sulfamethoxazole. Our study demonstrates important new insights into the genetic diversity, environmental persistence, and transmission of and potential effective therapy for Australian *Elizabethkingia* species.

KEYWORDS *Elizabethkingia*, MDR, multidrug resistance, nosocomial, MIC, minimum inhibitory concentration, antimicrobial resistance, AMR, comparative genomics, nosocomial infection

The genus *Elizabethkingia* (formerly *Chryseobacterium*) comprises a group of environmental bacteria that have traditionally been isolated from soil and water environments (1–4). As opportunistic pathogens, *Elizabethkingia* spp. can cause sporadic nosocomial outbreaks and infections in immunocompromised or at-risk individuals (1, 2, 5–8). Infections have been documented worldwide, such as those in the Central African Republic (9), Mauritius (10), Singapore (11), Taiwan (12), and the United

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States (6), suggesting a comprehensive global distribution that is yet to be fully described. To date, the largest outbreak was caused by community-acquired *Elizabethkingia anophelis* in Wisconsin, USA, from 2015 to 2016. A total of 66 individuals were infected, and the outbreak spread to the neighboring states of Illinois and Michigan (6). Comparative genomics characterized unique mutations by an integrative conjugative element (ICE) insertion in the *mutY* gene in all infecting strains, which may have accelerated the transmission of the outbreak clone. Additionally, a mutation in the *mutS* gene was identified in the only hypermutator strain, although the significance of this strain was unclear (6). Often, the source of *Elizabethkingia* species infection remains unclear, and routes of transmission are still to be defined (2, 6, 9, 12–16). However, previous investigations have suggested that shared water reservoirs within hospitals may be an overlooked reservoir of infection (1, 2, 17).

As an understudied pathogen, taxonomic assignment within the *Elizabethkingia* genus is ongoing. Recently, a formal taxonomic revision using whole-genome sequencing (WGS) has confirmed six *Elizabethkingia* species, consisting of *E. anophelis*, *E. meningoseptica*, *E. miricola*, *E. bruuniana*, *E. ursingii*, and *E. occulta* (3–5). It is also now recognized that *E. anophelis*, not *E. meningoseptica*, is the primary species causing human infection, with mortality rates currently estimated at 23 to 26% and 23 to 41%, respectively (4, 13, 18–20). Bacteremia, pneumoniae, sepsis, and meningitis are common clinical presentations with *E. anophelis* (7, 14, 18, 19). Similarly, *E. meningoseptica* infections present as neonatal meningitis and/or sepsis, but *E. meningoseptica* can also cause infections in most organ systems (8, 12). Risk factors associated with *Elizabethkingia* species infection consist of being male, having underlying chronic medical conditions, such as malignancy or diabetes mellitus, and admission to critical care or neonatal units (8, 12, 13, 18, 19, 21). The remaining members of the genus are thought to be much less prevalent in human disease; however, difficulties in accurately identifying *E. miricola*, *E. bruuniana*, *E. ursingii*, and *E. occulta* from clinical specimens have hindered appropriate recognition and characterization of these species (4).

Elizabethkingia species are considered resistant to carbapenems, cephalosporins, aminoglycosides, and most β-lactams even in combination with β-lactamase inhibitors (except for piperacillin-tazobactam), due to two unique metallo-β-lactamases (bla_{BlaB} and bla_{GOB}) and a unique extended-spectrum β-lactamase (ESBL) (bla_{CME}). Minocycline, levofloxacin, trimethoprim-sulfamethoxazole, and piperacillin-tazobactam are the most common antimicrobials that have been tested, with most strains demonstrating susceptibility to at least one or to various combinations of these antimicrobials (4, 6, 19–22). Due to the variations in susceptibility and the severity of infection, the most effective empirical therapy is still not known, highlighting the need for further MIC profiling (7, 19–21).

This study aimed to perform one of the largest comparative genomic analyses of *Elizabethkingia* species isolates to date, including isolates from Australia, a geographic area whose *Elizabethkingia* population is previously undescribed, as well as to assess the accuracy of identification of *Elizabethkingia* spp. with the Vitek MS version 3.2 database and to determine the MICs of clinical Australian *Elizabethkingia* isolates across 39 antimicrobials.

MATERIALS AND METHODS

Ethics statement. This project was reviewed by the chairperson of a National Health and Medical Research Council (NHMRC) and registered with The Royal Brisbane and Women's Hospital Human Research Ethics Committee (HREC) (EC00172) and was deemed compliant with the NHMRC guidance "Ethical Considerations in Quality Assurance and Evaluation Activities" 2014 and exempt from HREC review.

Isolates and initial identification. Twenty-two clinical *Elizabethkingia* species isolates collected in Queensland, Australia, over a 16-year period (2002 to 2018) were included in this study (Table 1). Isolates were collected by two methods. First, laboratory database storage records from multiple public and private laboratories in Queensland were searched for *Elizabethkingia* spp. or *Chryseobacterium meningoseptica*. Second, isolates identified by current laboratory identification systems as *Elizabethkingia* spp. were collected prospectively from both private and public pathology laboratories throughout the state of Queensland between January 2017 and October 2018. In both methods, isolates were stored at −80°C with low-temperature bead storage systems prior to being collected from storage and resurrected on 5%

TABLE 1 *Elizabethkingia* species isolates and associated identification information included in the current study

Isolate	Patient age (yrs)	Date collected	Sample type or collection site	Species identification using:	
				Vitek MS Knowledge Base 3.2	Whole-genome sequencing
EkQ1	1	2017	Sputum	*E. miricola*	*E. miricola*
EkQ3	43	2017	Sputum	*E. anophelis*	*E. anophelis*
EkQ4	78	2017	Blood	*E. meningoseptica*	*E. meningoseptica*
EkQ5	59	2017	Blood	*E. anophelis*	*E. anophelis*
EkQ6	17	2018	Bronchoalveolar lavage fluid	*E. anophelis*	*E. anophelis*
EkQ7	69	2018	Blood	*E. anophelis*	*E. anophelis*
EkQ8	0	2018	Urine	*E. anophelis*	*E. anophelis*
EkQ10	34	2018	Sputum	*E. miricola*	*E. miricola*
EkQ11[a]	**85**	**2018**	**Blood**	***E. miricola***	***E. bruuniana***
EkQ12	53	2018	Blood	*E. meningoseptica*	*E. meningoseptica*
EkQ13	1	2011	Sputum	*E. miricola*	*E. miricola*
EkQ15	16	2002	Bronchoalveolar lavage fluid	*E. anophelis*	*E. anophelis*
EkQ16	82	2017	Blood	*E. anophelis*	*E. anophelis*
EkQ17	66	2018	Blood	*E. anophelis*	*E. anophelis*
EkM1	Unknown	2018	Unknown	*E. anophelis*	*E. anophelis*
EkM2	Unknown	2018	Unknown	*E. anophelis*	*E. anophelis*
EkM3	Unknown	2014	Unknown	*E. anophelis*	*E. anophelis*
EkS1	80	2013	Blood	*E. anophelis*	*E. anophelis*
EkS2	82	2015	Blood	*E. anophelis*	*E. anophelis*
EkS3	74	2016	Blood	*E. anophelis*	*E. anophelis*
EkS4	73	2012	Blood	*E. anophelis*	*E. anophelis*
EkS5	66	2018	Dialysis fluid	*E. anophelis*	*E. anophelis*
EK1	NA	2019	Bathroom sink drain, oncology ward	*E. anophelis*	*E. anophelis*
EK2	NA	2019	Corridor sink drain, infectious disease ward	*E. anophelis*	*E. anophelis*
EK3	NA	2019	Hand-washing sink drain, oncology ward	*E. anophelis*	*E. anophelis*
EK4	NA	2019	Hand-washing sink, transplant ward	*E. anophelis*	*E. anophelis*
EK5	NA	2019	Bathroom handrail, transplant ward	*E. anophelis*	*E. anophelis*
EK6	NA	2019	Bathroom sink, transplant ward	*E. anophelis*	*E. anophelis*

[a]Strain EkQ11, marked in boldface, represents a species identification error according to Vitek MS Knowledge Base version 3.2.

horse blood agar (Edwards Group MicroMedia, Narellan, NSW, Australia). Single colonies were then double passaged on 5% horse blood agar and subjected to identification via Vitek MS Knowledge Base version 3.2 (bioMérieux, Murarrie, QLD, Australia). This database includes and can identify *E. anophelis*, *E. miricola*, and *E. meningoseptica*.

To investigate the epidemiology and transmission potential of *E. anophelis*, six environmental isolates were collected in 2019 from the Princess Alexandra Hospital, Brisbane, Australia, via swabbing various surfaces throughout the environment (Table 1). Specimens were plated onto 5% horse blood agar, and *Elizabethkingia* species colonies were double passaged to ensure purity and then subjected to identification via Vitek MS Knowledge Base version 3.2.

DNA extraction, whole-genome sequencing, and genome assembly. DNA was extracted using the DNeasy UltraClean microbial extraction kit (Qiagen, Chadstone, VIC, Australia) according to the manufacturer's instructions. Purified DNA was quantified using both the NanoDrop 3300 spectrophotometer and the Qubit 4 fluorometer (Thermo Fisher Scientific). Sequencing libraries were generated using the Nextera Flex DNA library preparation kit and sequenced on the MiniSeq system (Illumina, Inc., San Diego, CA, USA) on a high-output 300-cycle cartridge according to the manufacturer's instructions. Comparative genomic analyses were performed across a large *Elizabethkingia* data set ($n = 128$) (Table S1 in the supplemental material), including the 28 Australian genomes generated in the current study (Table 1), to assign species and to assess intraspecific and geographical relationships among strains. Publicly available *Elizabethkingia* Illumina reads ($n = 119$) were downloaded from the NCBI Sequence Read Archive database (January 2019), and *Elizabethkingia* species assemblies were downloaded from the GenBank database ($n = 109$). Publicly available Illumina reads were quality filtered with Trimmomatic version 0.38 (23) and subjected to quality control assessments with FastQC (24), followed by downsizing to 40× coverage using Seqtk (25). For assemblies without accompanying Illumina data, synthetic paired-end reads were generated with ART MountRainier-2016.06.05 (26). Genomes were limited to one representative per strain, and only sequence reads that were of high quality according to FastQC were included, to avoid errors in phylogenomic reconstruction ($n = 100$) (Table S1). The genomes were assembled using SPAdes version 3.13.0 (27) and annotated with Prokka version 1.13 (28) (Table S2).

Phylogenomic reconstruction. The comparative genomics pipeline SPANDx version 3.2 (29) was used under default settings to identify orthologous, biallelic, core-genome single-nucleotide polymorphism (SNP) and short insertion-deletion (indel) characters among the 128 *Elizabethkingia* genomes. *E. anophelis* strain NUHP1, *E. miricola* strain CSID_3000517120, *E. meningoseptica* strain G4120, and *E. bruuniana* strain G0146 (GenBank accession numbers NZ_CP007547.1, NZ_MAGX00000000.1, NZ_CP016378.1, and NZ_CP014337.1, respectively) were used as reference genomes for species-specific

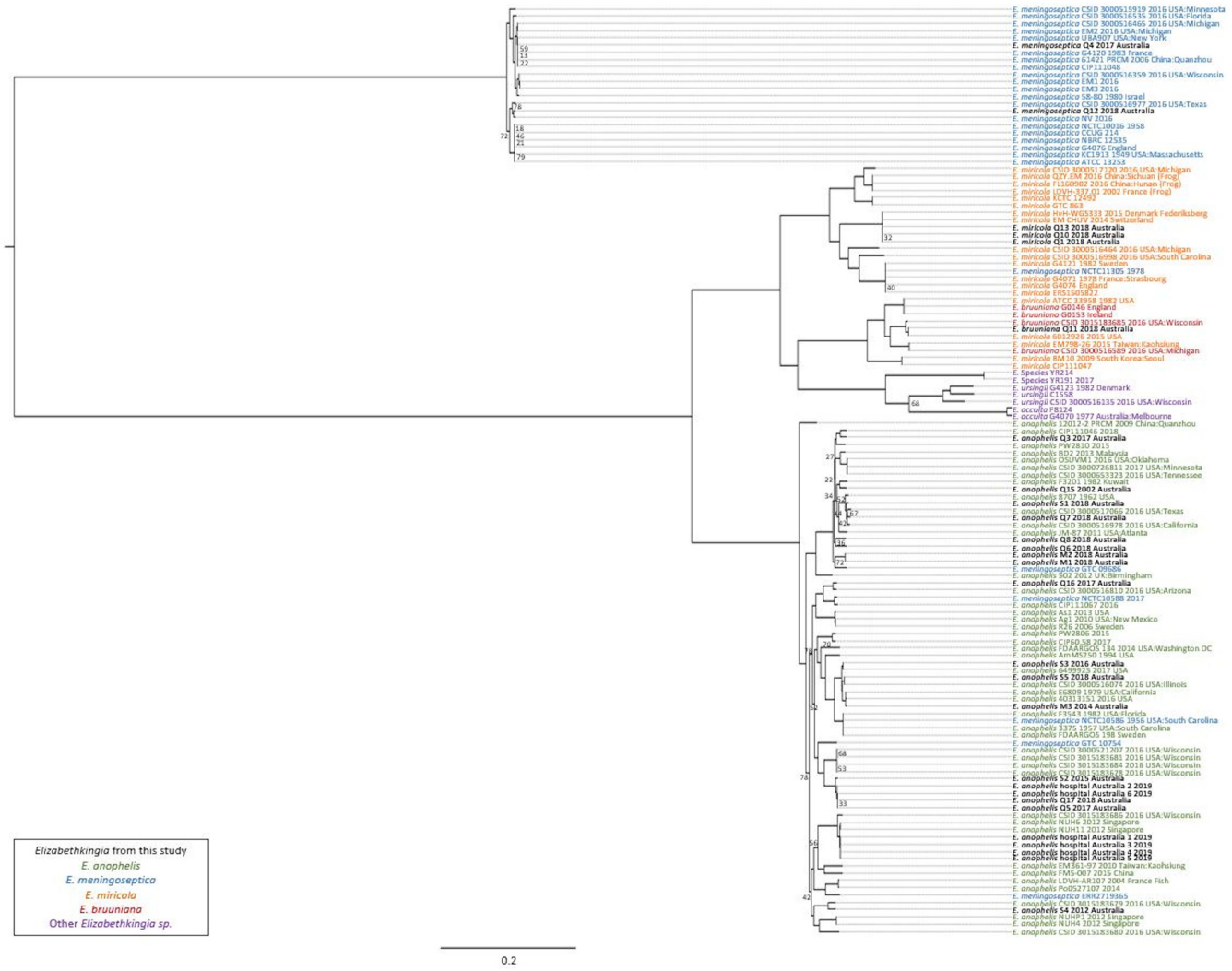

FIG 1 Global phylogenomic analysis of *Elizabethkingia* species genomes. Maximum-likelihood midpoint-rooted phylogeny. Branches returning bootstrap support of <0.8 are labeled. This phylogeny was reconstructed using 127,236 bialleleic, orthologous single-nucleotide polymorphisms identified among the 128 *Elizabethkingia* genomes.

SPANDx read-mapping alignment. Reference genome *E. anophelis* NUHP1 was used as the reference strain for the genus read mapping and alignment. Outputs from SPANDx were used to generate maximum-likelihood trees under the GTR+G model determined by jModelTest 2 (30) in RAxML version 8.2.12 (31) and visualized in FigTree version 4.0 (http://tree.bio.ed.ac.uk/software/figtree). From the 128 genomes, 127,236 SNPs were used to construct the *Elizabethkingia* genus phylogeny (Fig. 1). Within-species phylogenies were also constructed, using 121,827 SNPs from 71 genomes for *E. anophelis* (Fig. 2), 135,087 SNPs from 18 genomes for *E. miricola* (Fig. S1), 61,500 SNPs from 22 genomes for *E. meningoseptica* (Fig. S2), and 82,680 SNPs from 10 genomes for *E. bruuniana* (Fig. S3). All phylogenies were statistically tested with 1,000 bootstrap replicates. Branch support of less than 0.8 is shown in figures. To assess SNP and indel differences among closely related strains, the earliest-collected strain was used as the reference in SPANDx, and SNP and indel variants that had passed quality filtering were visualized in Tablet 1.19.09.03 (32) and Geneious Prime 2019 2.1 (33) (Table 2). For clonal isolates, ratios of nonsynonymous to synonymous evolutionary changes (dN/dS ratios) were calculated with MEGAx (Table S3).

MIC testing. *Elizabethkingia* species clinical isolates were subjected to broth microdilution to determine the MICs of 39 clinically relevant antimicrobials, consistent with or complementary to previous *Elizabethkingia* studies (Tables 3 and 4) (12, 19, 22, 34). Custom Gram-negative Sensititre MIC plates (ThermoFisher Scientific, Scoresby, VIC, Australia) were used according to the manufacturer's instructions. *E. bruuniana* isolate EkQ11 was excluded from MIC analyses due to poor growth. *Elizabethkingia* species isolates were compared against the European Committee on Antimicrobial Susceptibility Testing (EUCAST) pharmacokinetic-pharmacodynamic (PK-PD) nonspecies breakpoints (35) and the non-*Enterobacteriaceae* breakpoints according to the Clinical and Laboratory Standards Institute (CLSI) M100 guidelines (36–38). The MIC distributions for each antimicrobial are shown in Tables S4 and S5.

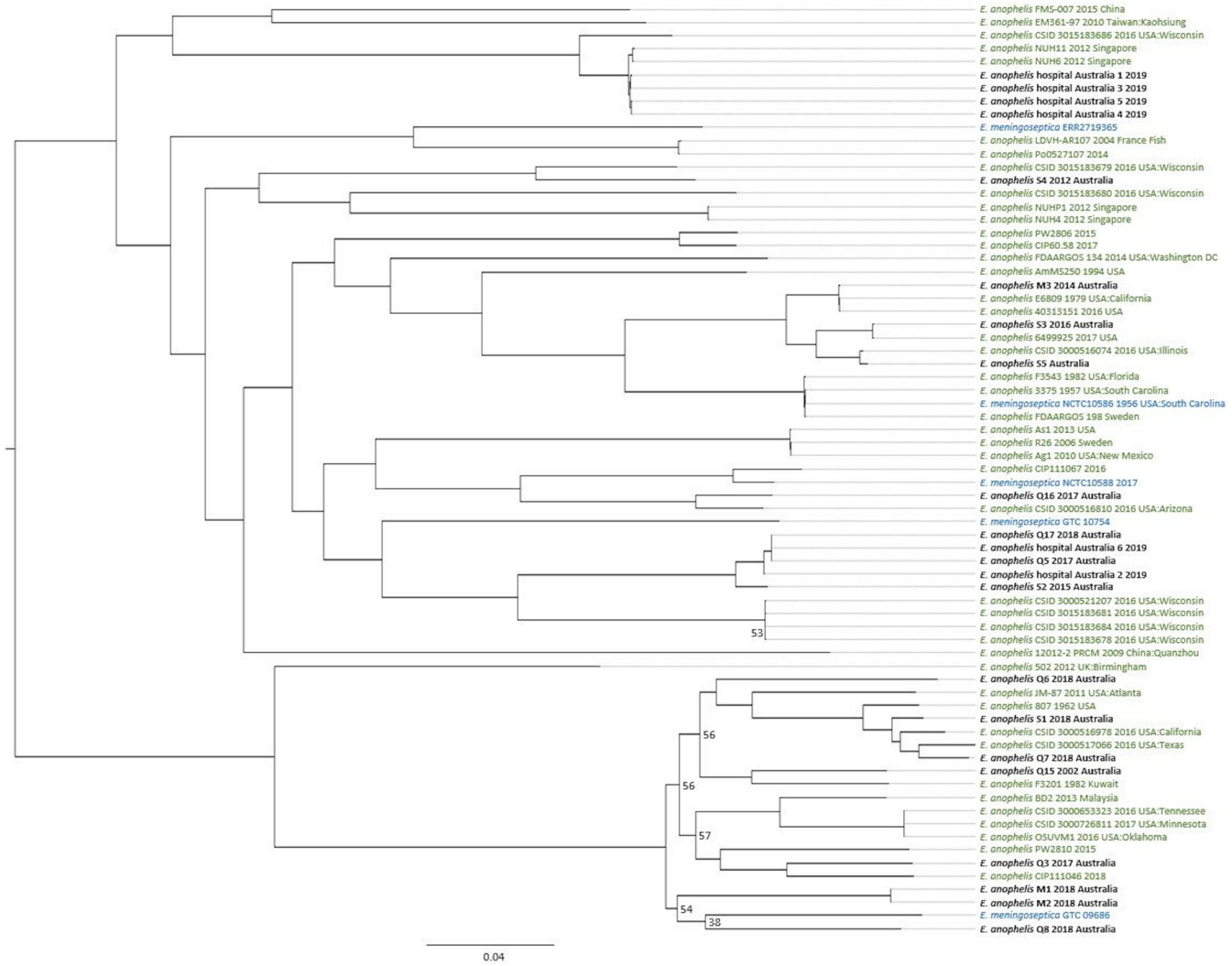

FIG 2 *Elizabethkingia anophelis* species-specific phylogenomic analysis. Maximum-likelihood midpoint-rooted phylogeny was reconstructed using 121,827 bialleleic, orthologous single-nucleotide polymorphisms identified among the 71 *E. anophelis* genomes. *E. anophelis* genomes correctly identified to species level are colored green, *Elizabethkingia meningoseptica* genomes incorrectly identified to species level are colored blue, and new *Elizabethkingia anophelis* genomes generated in this study are colored black. Branches returning bootstrap support of <0.8 are labeled.

***In silico* antimicrobial resistance (AMR) gene predictions.** Clinical *Elizabethkingia* species WGS data were subjected to ABRicate using both the CARD and NCBI databases to predict AMR genes (https://github.com/tseemann/abricate) and RAST for an alternative confirmation (34, 39, 40). Geneious prime 2019.2.1 and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to generate single-protein sequence alignments (33).

Data availability. Illumina sequence data for the 28 *Elizabethkingia* species genomes described in this study have been deposited in the NCBI in the SRA database under accession number SRP225137 and the BioProject database under accession number PRJNA576977 (BioSample accession numbers SAMN13016226 to SAMN13016247 and SAMN14081590 to SAMN14081595).

RESULTS

***Elizabethkingia* identification using comparative genomics versus mass spectrometry.** Phylogenomic reconstruction, including100 *Elizabethkingia* reference genomes collected internationally over the past 50 years, robustly identified the 22 clinical and 6 environmental Australian *Elizabethkingia* species isolates as *E. anophelis* ($n = 22$), *E. miricola* ($n = 3$), *E. meningoseptica* ($n = 2$), and *E. bruuniana* ($n = 1$) (Fig. 1; Table S1 in the supplemental material). Eleven identification errors were identified in the publicly available data set, including 2 identification errors within the *E. anophelis* clade, 5 within the *E. bruuniana* clade, and 1 within the *E. miricola* clade (Fig. 1). Additionally, com-

TABLE 2 Single-nucleotide polymorphism and deletion differences between strains of the clonal cluster of clinical and environmental *Elizabethkingia anophelis* isolates[a]

Mutation	EkQ5 2018	EkQ17 2018	EK6 2019	Protein affected	Effect
SNP	G	A	A	Hypothetical Protein	A10T
SNP	G	A	A	Efflux pump membrane transporter (bepE)	S416R
SNP	C	T	T	3-oxoacyl-[acyl-carrier-protein] synthase 2 (fabF)	R303H
SNP	T	C	C	Penicillin binding protein E (pbpE_7)	*762W (+ 279aa)
SNP	A	T	T	Sugar transporter	*133R (+ 133aa)
SNP	C	T	C	Hypothetical Protein	E168K
SNP	C	T	T	Protease (S41 family)	T161I
SNP	G	A	G	B-galactosidase (lacZ_2)	no change
DEL	CT	C	C	Hypothetical Protein	R65E (+ 9aa)

[a]Single-nucleotide polymorphism and deletion differences between strains of the clonal cluster of clinical and environmental *Elizabethkingia anophelis* isolates. Clinical isolates EkQ5 (earliest-collected and reference strain) and EkQ17 were collected from two different transplant patients, while Ek6 was collected from a shared handwashing sink on the transplant ward. Gray shading shows no differences, green shows similarities between EkQ17 and EK6, and blue highlights unique changes. The proteins affected by each mutation and the resulting amino acid changes are also shown. SNP, single-nucleotide polymorphism; DEL, deletion.

parison of Vitek MS Knowledge Base version 3.2 with genomic species assignments of the Australian isolates resulted in one identification error from the Vitek MS, incorrectly identifying an *E. bruuniana* isolate as *E. miricola* (Table 1).

Australian *Elizabethkingia* and global relatedness. Australian *Elizabethkingia* spp. displayed no distinct phylogeographical signal within the genus phylogeny, as they disseminated across the phylogenetic tree (Fig. 1). No Australian *Elizabethkingia* isolate was identical to a previously described isolate, with those appearing to be nearly identical in the phylogenies separated by 16 to 284 SNPs (Fig. 1 and 2; Fig. S1 to S3). Australian *E. anophelis* isolates are not closely related to Wisconsin, USA, outbreak strains (Fig. 1 and 2). Clinical isolates EkQ17, EkQ5, and EkS2 and environmental isolates EK2 and EK6 branched off the Wisconsin, USA, outbreak cluster, diverging as a distantly related unique lineage separated by an estimated 20,400 SNPs and 500 indels using CSID_3015183681 as the reference strain. The truncations of the C termini of MutY and MutS, characteristic of the outbreak and hypermutator strains, respectively, were not identified in Australian strains from the amino acid alignment of these proteins. Furthermore, the 2019 hospital environmental isolates EK1, EK3, EK4, and EK5, collected from various wards' hand-washing sinks or toilet environments, were from the same hospital as the EkQ5-EkQ17-EK6-EK2 clade. The EK hospital isolates from this study are separated from the 2012 Singaporean isolates NUH6 and NUH11 by 656 to 867 SNPs and 41 to 72 indels, respectively, and share a clade with 2016 outbreak isolate CSID_3015183686.

Evidence of *E. anophelis* nosocomial transmission. Two instances of recent closely related Australian *E. anophelis* isolates were identified on two separate lineages by phylogenetic analysis (Fig. 2). In the first instance, two isolates, EkM1 and EkM2, were collected from the same patient 1 month apart, branching as a unique lineage with clinical isolate EkQ8 from a patient in a different hospital (Fig. 2).

In the second instance, diverging from the Wisconsin outbreak cluster in the *E. anophelis* phylogeny are five epidemiologically linked clinical isolates, EkQ5, EkQ17, EkS2, and hospital environmental isolates EK2 and EK6 (Fig. 2). SNP and indel comparisons between clinical strains EkQ5 and EkQ17, from two different patients admitted into the same transplant ward 9 months apart in 2018, revealed a difference of 8 SNPs

TABLE 3 MIC data for Australian clinical *Elizabethkingia anophelis* isolates against clinically relevant antimicrobials

Antimicrobial[a]	Range Tested (μg/mL)	MIC_{50} (μg/mL)	% S	% I	% R	% S	% I	% R
Cephalexin	4-64	>64						
Cefazolin	0.25-32	>32	6.25		93.76			
Cefuroxime	1-16	>16			100		100	
Cefoxitin	8-512	512						
Cefotaxime	0.03-8	>8	6.25		93.76	6.25	93.75	
Ceftazidime	0.12-16	>16						100
Ceftriaxone	0.03-4	>4			100		100	
Cefepime	0.06-16	>16	6.25	12.5	81.25	18.75	81.25	
Ceftaroline	0.25-16	>16			100			
Ceftolozane/tazobactam[b]	0.25-16	16	6.25	31.25	62.5			
Amikacin	1-64	64				6.25	50	43.75
Gentamicin	0.25-16	>16					50	50
Tobramycin	0.25-16	>16						100
Meropenem	0.015-16	>16	6.25		93.7	6.25		93.75
Doripenem	0.03-8	>8						
Etrapenem	0.015-4	>4		6.25	93.76			
Imipenem	0.06-16	>16			100	6.25		93.75
Doxycycline	0.12-64	8				68.75	31.25	
Minocycline	0.25-32	1				100		
Tigecycline	0.12-8	2		12.5	87.5			
Ciprofloxacin	0.015-4	2	6.25	50	43.75	75	25	
Levofloxacin	0.03-4	1	56.25	37.5	6.25	100		
Amoxicillin	2-32	>32			100			
Ampicillin	2-32	>32			100			
Amoxicillin/clavulanic acid[c]	4-128	>128			100			
Ampicillin/sulbactam[d]	8-128	>128			100			
Temocillin	2-64	>64						
Piperacillin/tazobactam[b]	1-64	>64		31.25	68.75	31.25	68.75	
Vancomycin	0.12-64	64						
Teicoplanin	0.12-64	64						
Azithromycin	4-64	32						
Aztreonam	0.15-16	>16			100		100	
Trimethoprim	0.5-16	>16						
Trimethoprim/sulfamethoxazole	0.12-16	8:152				75		25
Chloramphenicol	2-256	256				6.25	12.50	81.25
Colistin	0.25-8	>8						
Polymyxin	0.25-8	>8						
Rifampicin	0.12-64	0.5						

[a]MIC data derived from broth microdilution testing of the 16 Australian clinical *E. anophelis* isolates against 39 clinically relevant antimicrobials. Pharmacokinetic-pharmacodynamic (non-species-specific) breakpoints applied from EUCAST clinical breakpoint tables (version 9.0) are shown in columns with yellow shading, and non-*Enterobacteriaceae* breakpoints applied from CLSI M100-29 (2019) are shown in columns with blue shading. Shaded blue and yellow cells indicate that no breakpoint is currently available for this antimicrobial within these schemes. S, susceptible; I, susceptible with high exposure (EUCAST definition) or intermediate (CLSI definition); R, resistant.

[b]Tazobactam concentration fixed at 4 mg/liter.

[c]Clavulanic acid concentration fixed at 2 mg/liter.

[d]Sulbactam concentration fixed at 4 mg/liter.

TABLE 4 MIC data for Australian clinical *Elizabethkingia meningoseptica* and *Elizabethkingia miricola* isolates against clinically relevant antimicrobials

Antimicrobial[a]	Range Tested (μg/mL)	% S	% I	% R	% S	% I	% R
Cephalexin	4-64						
Cefazolin	0.25-32			100			
Cefuroxime	1-16			100		100	
Cefoxitin	8-512						
Cefotaxime	0.03-8			100		100	
Ceftazidime	0.12-16			100			100
Ceftriaxone	0.03-4			100		100	
Cefepime	0.06-16			100			100
Ceftaroline	0.25-16			100			
Ceftolozane/tazobactam[b]	0.25-16			100			
Amikacin	1-64				33.3	66.6|50	50
Gentamicin	0.25-16				50|50		66.6|50
Tobramycin	0.25-16						100
Meropenem	0.015-16			100			100
Doripenem	0.03-8						
Etrapenem	0.015-4			100			
Imipenem	0.06-16			100			100
Doxycycline	0.12-64				66.6|100	33.3	
Minocycline	0.25-32				100		
Tigecycline	0.12-8			100			
Ciprofloxacin	0.015-4	100	100		100		
Levofloxacin	0.03-4	100		100	100		
Amoxicillin	2-32			100			
Ampicillin	2-32			100			
Amoxicillin/clavulanic acid[c]	4-128			100			
Ampicillin/sulbactam[d]	8-128			100			
Temocillin	2-64						
Piperacillin/tazobactam[b]	1-64			100		100	
Vancomycin	0.12-64						
Teicoplanin	0.12-64						
Azithromycin	4-64						
Aztreonam	0.15-16			100		100	
Trimethoprim	0.5-16						
Trimethoprim/sulfamethoxazole	0.12-16				33.3|50		66.6
Chloramphenicol	2-256				100	100	
Colistin	0.25-8						
Polymyxin	0.25-8						
Rifampicin	0.12-64						

[a]MIC data derived from broth microdilution testing of the 2 *E. meningoseptica* (blue font) and 3 *E. miricola* (orange font) Australian clinical isolates against 39 clinically relevant antimicrobials. Pharmacokinetic-pharmacodynamic (non-species-specific) breakpoints applied from EUCAST Clinical Breakpoint Tables (version 9.0) are shown in columns with yellow shading, and non-*Enterobacteriaceae* breakpoints applied from CLSI M100-29 (2019) are shown in columns with blue shading. Shaded blue and yellow cells indicate that no breakpoint is currently available for this antimicrobial within these schemes. S, susceptible; I, susceptible with high exposure (EUCAST definition) or intermediate (CLSI definition); R, resistant.

[b]Tazobactam concentration fixed at 4 mg/liter.

[c]Clavulanic acid concentration fixed at 2 mg/liter.

[d]Sulbactam concentration fixed at 4 mg/liter.

and 1 indel. Epidemiologically, EkQ5, EkQ17, and EkS2 appear to be linked to a single environmental source within the transplant ward.

Mutational differences between EkQ5-EkQ17 and EK6 were mostly nonsynonymous in nature, consistent with adaptive evolution (dN/dS ratios presented in Table S3). Of the two SNPs separating EkQ17 and EK6, one resulted in a missense mutation (resulting in a change from E to K at position 168 [E168K]) in a hypothetical protein (Ek00046). Between EkQ5 and EK6, 4 SNPs resulted in missense mutations and 2 caused nonsense mutations in penicillin-binding protein E (PbpE) and a sugar transporter protein that increased protein length, likely leading to altered or lost protein function (Table 2). In addition, the indel mutation accrued by EkQ5 resulted in a frameshift mutation that elongated a hypothetical protein (Ek02802) by 9 residues, potentially altering its function.

Another hospital environmental isolate, EK2, was linked to the EkQ5-EkQ17-EK6 clade according to phylogenetic analysis, differing by 38 SNPs and 16 indels (Fig. 2). This isolate was collected in 2019 from a sink drain in the infectious disease ward adjacent to the transplant ward where EkQ5, EkQ17, and EK6 were isolated. A more distantly related clinical isolate, EkS2, also clustered within the same clade as the EkQ5-EkQ17-EK6-EK2 isolates but differed from these isolates by 3,552 SNPs and 120 indels. Consistent with the phylogenomic findings, EkS2 was not epidemiologically linked to the EkQ5-EkQ17-EK6-EK2 isolates, being isolated from a patient admitted to a different hospital in 2015.

MICs. A total of 39 clinically relevant antimicrobials were tested across the 22 clinical *E. anophelis*, *E. miricola*, and *E. meningoseptica* isolates. Modal MICs were relatively consistent within and between species and predominantly sat on the higher end of the ranges tested (Tables S4 and S5). *Elizabethkingia* does not have a defined clinical breakpoint, and therefore, species were examined against the EUCAST nonspecies and CLSI non-*Enterobacteriaceae* PK-PD breakpoints. The EUCAST breakpoints suggest that Australian strains have the greatest resistance to cephalosporins, carbapenems, and penicillins, even in combination with β-lactamase inhibitors (amoxicillin-clavulanic acid, piperacillin-tazobactam, and ampicillin-sulbactam). Furthermore, the CLSI breakpoints suggest high levels of resistance to amikacin, gentamicin, tobramycin, and chloramphenicol. From the MICs (Tables 3 and 4), only a select few antimicrobials had modal MICs in the lower range, including tetracyclines (doxycycline, 2 μg/ml, and minocycline, 0.5 to 1 μg/ml), fluoroquinolones (ciprofloxacin, 0.25 μg/ml, and levofloxacin, 0.25 μg/ml) and trimethoprim-sulfamethoxazole (1 μg/ml) (Tables 3 and 4). Only minocycline achieved 100% susceptibility across all *E. anophelis* strains using the CLSI non-*Enterobacteriaceae* PK-PD breakpoints. Rifampin and azithromycin do not have corresponding EUCAST or CLSI PK-PD breakpoints; however, their respective modal MICs are also on the lower end of the ranges tested, suggesting the potential for susceptibility. Vancomycin also lacks corresponding EUCAST or CLSI PK-PD breakpoints, but based on the MICs observed (range, 8 to 64 μg/ml), it is expected the isolates are nonsusceptible. One *E. anophelis* isolate, EkQ6, was responsible for the low MICs observed across the antimicrobials tested, remaining susceptible to cephalosporins and carbapenems, in addition to the fluoroquinolones, tetracyclines, and trimethoprim-sulfamethoxazole.

***In silico* AMR gene analysis.** All 22 clinical *Elizabethkingia* species genomes carried all three previously described β-lactamases that are characteristic of *Elizabethkingia*. The chromosomal extended-spectrum β-lactamase bla_{CME} encodes cephalosporin and β-lactamase activity, while metallo-β-lactams bla_{BlaB} and bla_{GOB} encode activity against carbapenemases and penicillin–β-lactamase combinations. The metallo-β-lactamase bla_{BlaB} carried a missense mutation of $bla_{BlaB}^{\Delta T16A}$ in EkQ6. Three *E. miricola* isolates and the *E. bruuniana* isolate carried an AmpC variant with 94 to 95% sequence similarity to AmpC identified in *E. anophelis* and *E. miricola* genomes (accession numbers CP006576, CP007547, and CP011059). All isolates carried a conserved AmpG, with three strains exhibiting 5′-end truncations $AmpG^{M1\_A243del}$ and one strain exhibiting $AmpG^{M1\_A3del}$. All isolates also carried tetracycline resistance gene *tet*(X), chloramphenicol resistance

gene *catB*, and aminoglycoside resistance gene *aadS*. Except for that of *E. bruuniana* EkQ11, all Australian *Elizabethkingia* species genomes carried the vancomycin resistance protein VanW.

DISCUSSION

Elizabethkingia spp. have caused serious nosocomial infections and outbreaks globally, and yet, they have received little attention to date. This study aimed to fill knowledge gaps surrounding the diversity, origin, and transmission events of clinical and environmental *Elizabethkingia* species isolates from Australia, a previously unstudied population.

***Elizabethkingia* identification using comparative genomics versus mass spectrometry.** The 28 Australian *Elizabethkingia* isolates were identified as *E. anophelis*, *E. meningoseptica*, *E. miricola*, and *E. bruuniana*, with *E. anophelis* as the primary infecting species in Australia, similar to recent global reports (7, 18, 21). Despite a previous review of identification failure using mass spectroscopy for species other than *E. anophelis* and *E. meningoseptica* (4), Vitek MS Knowledge Base version 3.2 performed reliably in this study, with 96.2% accuracy. *E. bruuniana* (one isolate) was the only species that could not be accurately identified, instead being identified as the sister species *E. miricola*. This could be due to the species not yet being present in the database, or perhaps to *E. miricola* and *E. bruuniana* being variations of the same species, as many previous identification errors were seen in the genus phylogeny (Fig. 1). Nevertheless, identification of *E. miricola* should be taken with caution until the database has been upgraded with the capabilities to differentiate between the sister species.

Australian *Elizabethkingia* and global relatedness. Phylogenomic analyses of Australian clinical isolates revealed dispersal throughout the tree and unique lineages in some strains. Others branched with previously identified geographically diverse isolates from both clinical and environmental settings (Fig. 1). Recently, DNA-DNA hybridization and average nucleotide identity have allowed the reclassification of *E. miricola* strains ATCC 33958, BM10, and EM798-26 to *E. bruuniana* (3, 21, 41). Further to these corrections, using comparative genomics, we suggest the reclassification of *E. miricola* strains 6012926 and CIP111047 to *E. bruuniana*, *E. meningoseptica* strains NCTC10588 and NCTC10586 to *E. anophelis*, and lastly, *E. meningoseptica* NCTC11305 to *E. miricola* (Fig. 1). Evidence from past studies has described the structure of *E. anophelis* phylogenies as consisting of two and six major clades (6, 42); in this study, we identified six lineages, yet as sampling continues, this may expand (Fig. 2).

Several *E. anophelis* isolates from this study cluster phylogenetically with the Wisconsin outbreak strains from 2016, the most pathogenic *Elizabethkingia* outbreak to date (6). Outbreak and hypermutator strains have been characterized by their ICE insertions and truncations at the C terminus in both the MutS and MutY protein sequences, respectively (6). The MutS and MutY protein sequences in our clinical isolates aligned with few nonsynonymous amino acid changes and no truncations, and therefore, it is unlikely that the Australian clinical isolates would display the outbreak characteristics or phenotype suspected to be responsible for the increased pathogenesis of the Wisconsin strains or the hypermutator phenotype identified in one Wisconsin strain. Several pathogenicity islands were identified in both Australian and Wisconsin *E. anophelis* strains, suggesting they may play an important role in the species survival or pathogenesis.

Potential nosocomial transmission of *E. anophelis* in a transplant ward. A recent case of hospital-acquired *E. anophelis* infection was suggested by the identification of a clonal cluster comprised of clinical and environmental isolates in this study. A pair of Australian *E. anophelis* clinical isolates, EkQ5 and EkQ17, collected almost a year apart in 2018 from two patients on the transplant ward, were characterized as differing by only eight SNPs and one deletion. Additionally, it was found that the hospital environmental sample collected from a hand-washing sink in the same transplant ward in late 2019 only differed from clinical sample EkQ5 by six of the same eight SNPs and the one deletion (Table 2). The combination of clinical and environmental genomic data with

such low genetic diversity suggests that these strains were transmitted via the common reservoir of the hand-washing sink, given the extended time frame between patient infection and environmental collection. Nearly identical isolates have been described previously within *E. anophelis*, such as environmentally collected isolates OSUVM-1 and -2 (43), hospital outbreak strain NUHP (44), and Wisconsin CSID strains (6), suggesting that low genetic variation is not unusual among *E. anophelis* infections. The relatedness of sink or toilet environment hospital isolates EK4 and EK5 from the transplant ward to EK1 and EK3 from the oncology ward suggests that another transmission event may have also taken place, despite not identifying a related clinical isolate. Interestingly, these hospital environmental isolates (EK1, -3, -4, and -5) formed a clonal cluster and appear to share similarity to two 2012 Singaporean clinical isolates, NUH6 and NUH11, which were also isolated from hospital environments (11).

Additional studies have reported contaminated communal water sources as a reservoir for *Elizabethkingia* species infections within hospitals (1, 17), with handwashing stations in a pediatric intensive care unit the source of several *Elizabethkingia* species infections in Singapore, where staff transmitted the infection after handwashing (2). Although direct human-to-human transmission is seen in many other nosocomial infections (45, 46) and vertical transmission has been reported in *E. anophelis* (47), the role human-to-human transmission has in *Elizabethkingia* infections still remains unclear. However, given the severity of the infections, known patient risk factors, and the suggested longevity of the bacteria in the environment, the potential for horizontal transmission should not be overlooked.

MIC testing. The MIC data generated in this study confirm that, like those in previous studies, the Australian clinical *Elizabethkingia* species isolates (with the exception of isolate EkQ6) (Tables S4 and S5 in the supplemental material) are resistant to many antimicrobial classes, including cephalosporins, carbapenems, and aminoglycosides (Tables 3 and 4) (12, 19, 20, 22, 48). From the literature, there is some variation in *E. anophelis* antimicrobial resistance profiles among isolates from the United States, Southeast Asia, and South Korea, while Australian isolates appear to phenotypically express some marked differences. For example, approximately 75 to 100% of *E. anophelis* isolates were reported to be resistant to trimethoprim-sulfamethoxazole (6, 19–22), while 75% of Australian strains remained sensitive. Additionally, 88 to 95% of isolates were susceptible to piperacillin-tazobactam (6, 19, 21, 22), while 68 to 70% of Australian and South Korean (20) isolates were resistant. Vancomycin has been suggested as a potential therapy for *E. meningoseptica* infections; therefore, we screened our *E. anophelis* strains against vancomycin and additional antimicrobials with Gram-positive activity, such as teicoplanin. Despite the recommendations for vancomycin use in *Elizabethkingia* infections (4, 20, 49, 50), our data show resistance among Australian clinical isolates, as the MICs were on the high end of the range tested and all isolates except *E. bruuniana* (EkQ11) carried the *vanW* gene. This is the first set of MIC data for teicoplanin, and with a modal MIC of 32 μg/ml, these strains appear to be resistant. Similar to the Wisconsin outbreak strains (6), Australian *Elizabethkingia* species strains may be susceptible to azithromycin, as the modal MIC of 4 μg/ml is on the lower end of the range tested. Although doxycycline testing against *E. anophelis* is not often reported in the literature, others have found their strains to be highly susceptible, unlike in our study (22). EUCAST breakpoints suggest that 6.25% and 43.75% of Australian *E. anophelis* isolates are resistant to levofloxacin and ciprofloxacin, respectively. Variability in fluoroquinolone susceptibility has also been observed in the majority of Southeast Asian and United States strains (6, 19–22). It is clear that numerous antimicrobials have been tested across *E. anophelis* isolates in previous studies, although susceptibility to multiple antimicrobial classes like that observed in EkQ6 has not been reported previously. Further testing of *E. anophelis* isolates from Australia and abroad would determine whether this type of sensitivity is unique to a subset of Australian strains or is present globally.

***In silico* AMR gene analysis.** Antimicrobial resistance (AMR) genes bla_{BlaB}, bla_{GOB}, and bla_{CME} were identified within the genomes of all clinical Australian *Elizabethkingia* isolates, linking directly to their observed MIC profiles. All isolates except EkQ6 were resistant to cephalosporins and β-lactams, attributed to bla_{CME}, and to carbapenemases and penicillin–β-lactamase combinations, attributed to bla_{BlaB} and bla_{GOB} (4, 51–53). Additionally, in conjunction with these three beta-lactamase genes, AmpC has been identified previously in a few *Elizabethkingia* genomes (*E. anophelis* and *E. miricola*) and was identified here in three *E. miricola* genomes and an *E. bruuniana* genome with high sequence similarity. Resistance attributed to AmpC and its exact role in *Elizabethkingia* species are still to be described, as there are no observed differences in susceptibility profiles of strains harboring AmpC to date (20). However, fluoroquinolone resistance varied, as described above, and is mediated by a single-step amino acid substitution (Ser83Ile or Ser83Arg) in GyrA (19, 21, 54) that was not identified in any of the Australian clinical *Elizabethkingia* isolates. The absence of the mutation has also been reported recently for a single isolate in Taiwan (22). Previous studies have linked DNA topoisomerase IV to an assistance-type role in fluoroquinolone resistance for *Elizabethkingia* spp. (22, 54), although this was not identified in our clinical isolate collection either. Phenicol resistance conferred by *catB* genes has been identified in *Elizabethkingia* previously, with all Australian clinical isolates harboring this gene (55, 56). Aminoglycoside and tetracycline resistance genes *aadS* and *tetX* were also present in all genomes, the former likely responsible for the observed aminoglycoside resistance (Tables 3 and 4), with similar genes shown to confer resistance in *Elizabethkingia* species (56, 57). Although *tetX* was present in all isolates, nearly ubiquitous susceptibility to tetracyclines was observed in MIC profiles, suggesting that *tetX* alone does not confer tetracycline resistance in some *Elizabethkingia* species or that it could be a silent gene, although it has been identified as a resistance mechanism in *E. coli* (56, 58).

In addition, clinical *E. anophelis* isolate EkQ6 carried several mutations not commonly described in bla_{BlaB} and *topA* (21, 22, 50, 56) and yet remained susceptible to cephalosporins, carbapenems, tetracyclines, and fluoroquinolones. The substitutions and deletions, respectively, may or may not be linked to the susceptibility of this isolate. Comparative genomics, including more susceptible isolates like EkQ6, would provide great insight into the intrinsic antimicrobial resistance mechanisms of *Elizabethkingia* species (50, 59, 60).

Potential antimicrobial therapy for *Elizabethkingia* spp. In this study, Australian isolates appear to be susceptible to fluoroquinolones, tetracyclines, and trimethoprim-sulfamethoxazole. Only levofloxacin and minocycline demonstrated 100% susceptibility using CLSI PK-PD breakpoints. Fluoroquinolone treatment alone has proven to be successful in *Elizabethkingia* species infections (61), but some recommend combination therapy (62) in order to mitigate high-level fluoroquinolone resistance for those susceptible to single-step mutations. From our and other studies, susceptibility is clearly strain dependent. Our findings suggest that rifampin (63) or azithromycin could also be effective antimicrobials, although this would require further testing. With this in mind and the recent success of newer antimicrobials against MDR Gram-negative bacteria (64–66), it would be of value to further test *Elizabethkingia* spp. against newer antimicrobials, such as cefiderocol (67). Due to the pathogenic nature of *Elizabethkingia* infections, therapy should always be guided by patient condition and MIC data.

Conclusions. This study has characterized the diversity of Australian *Elizabethkingia* spp. genotypically and phenotypically using comparative genomics and antimicrobial resistance. We have revealed significant strain diversity in Australia and have shown that Vitek MS Knowledge Base version 3.2 can accurately identify *E. anophelis*, *E. meningoseptica*, and *E. miricola* species but is not yet able to correctly identify *E. bruuniana*. Furthermore, genomic exploration has provided insight into the breadth of the intrinsic MDR nature of *Elizabethkingia* species infections and revealed a potential reservoir of infection within a hospital setting, where two patients were infected with nearly identical strains. Antimicrobial resistance data suggest that clinical isolates are

susceptible to fluoroquinolones, tetracyclines, and trimethoprim-sulfamethoxazole. Specifically, minocycline and levofloxacin showed suitable efficacy against *Elizabethkingia* isolates *in vitro*, although further clinical studies are required to define optimal therapy.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.01 MB.

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