First reported isolation of an emerging opportunistic pathogen (*Elizabethkingia anophelis*) from hospital water systems in Greece

Maria A. Kyritsi, Varvara A. Mouchtouri, Spyros Pournaras and Christos Hadjichristodoulou

ABSTRACT

After the *Elizabethkingia anophelis* outbreak in Wisconsin, USA, an active search for the detection of the microorganism in hospital water systems from Central and Northern Greece was performed from June to December 2016. In total, 457 water samples from 11 hospitals were analyzed. *Elizabethkingia* spp. was detected in three samples collected from two hospitals, both of which are located in Northern Greece. Two of the three isolated strains were identified as *Elizabethkingia anophelis*. No cases of *Elizabethkingia* infection were reported in either hospital during 2016. This is the first reported isolation of the pathogen in water supply systems in Greece.

Key words | *Elizabethkingia anophelis*, Greece, hospitals, water systems

Maria A. Kyritsi
Varvara A. Mouchtouri
Christos Hadjichristodoulou (corresponding author)
Department of Hygiene and Epidemiology, Medical School,
University of Thessaly,
Larissa,
Greece
E-mail: *xhatzi@med.uth.gr*

Spyros Pournaras
Department of Microbiology, Medical School,
National & Kapodistrian University of Athens,
Athens,
Greece

INTRODUCTION

Members of the genus *Elizabethkingia* are Gram-negative, non-motile, non-fermenting, aerobic bacteria. The genus belongs to the phylum *Bacteroidetes* and the family *Flavobacteriacae* and initially, comprised four species: *Elizabethkingia meningoseptica*, *Elizabethkingia anophelis*, *Elizabethkingia miricola*, and *Elizabethkingia endophytica* (Kim *et al.* 2005; Kämpfer *et al.* 2015). Both *E. meningoseptica* and *E. miricola* were previously classified to the genus *Chryseobacterium* (Vandamme *et al.* 1994; Kim *et al.* 2005). Recently, using whole genome sequencing (WGS) *E. endophytica* was proved to be a subjective synonym of *E. anophelis* (Doijad *et al.* 2016). *Elizabethkingia* is a microorganism with currently vague pathophysiology, transmission, and reservoir (Centers for Disease Control and Prevention 2016). All species of the genus are commonly found in the environment (soil, water, and plants). In particular, *E. anophelis* is abundant in the midgut of the mosquito *Anopheles gambiae* (Lau *et al.* 2016). The microorganism may colonize hospital environment, is highly persistent to decontamination measures, thus contaminating medical solutions and devices (Moore *et al.* 2016). Recent studies have proposed that hospital water supply systems possibly act as a reservoir, being responsible for long-term transmission of the microorganism in the hospital environment (Lau *et al.* 2015; Moore *et al.* 2016).

E. meningoseptica mainly causes healthcare-associated infections in immunocompromised patients as well as neonatal meningitis and sepsis (Lau *et al.* 2015, 2016; Tai *et al.* 2016). Infections caused by *E. meningoseptica* are often very severe, displaying high death rates. The existing comorbidities and immunosuppression of these patients in combination with the multidrug-resistant profile of the microorganism (Lau *et al.* 2016) contribute to the fatal outcome of the infection.

E. anophelis has been widely known since the outbreak in Wisconsin, USA, that was attributed to the microorganism, which accounted for 67 cases and 18 deaths from 01.11.2015 to 11.01.2017 (Centers for Disease Control and

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Prevention 2016; Wisconsin Department of Health Services 2017). *E. anophelis* usually causes pneumonia and bacteremia with high rates of mortality (Lau *et al.* 2016). Recently published data indicate that the outbreak was caused by a single, highly pathogenic strain with 13 characteristic genomic regions (Perrin *et al.* 2017). Consequently, *Elizabethkingia* can be considered an emerging opportunistic pathogen and although the majority of the strains are primarily identified as *E. meningoseptica*, they are in fact *E. anophelis*, as 16S rRNA gene sequencing and WGS indicates (Breurec *et al.* 2016; Nicholson *et al.* 2016; Eriksen *et al.* 2017).

To the best of our knowledge until presently, the study of Arvanitidou *et al.* (2003) is the only one supporting the presence of the species in hemodialysis water and dialysate in renal units in Greece. The study denoted the presence of *Chryseobacterium meningosepticum* (presently *E. meningoseptica*) in hemodialysis water and dialysate at a frequency of 14.9%.

Urged by the outbreak of *E. anophelis* in Wisconsin, USA, the source and transmission of which is still unknown (Centers for Disease Control and Prevention 2016; Navon *et al.* 2016), and by lately published research that traced *E. anophelis* in the hospital water supply system (Breurec *et al.* 2016), we decided to analyze all water samples that we received from hospitals from June to December 2016 for the presence of *Elizabethkingia* spp. This is the first report for the presence of *E. anophelis* in hospital water supply systems in Greece.

METHODS

A total of 457 tap water samples from 11 hospitals of Northern and Central Greece were analyzed at the Laboratory of Hygiene and Epidemiology of the University of Thessaly, Greece. All samples were collected without disinfecting the tap before sample collection. Water samples were collected and transported to the laboratory according to the national standards that are based on ISO 5667-5. A total of 100 mL of water was filtered by using 0.45 μm filter membranes (EZ-Pak Filters 0.45 μm, 47 mm white gridded, Merck, Millipore), and the membranes were incubated on nutrient and MacConkey agar, for each sample, in ambient air at 37°C for 48 hours. Samples where Gram-negative, oxidase-positive (Bactident-Oxidase strips, Merck), catalase-positive colonies were isolated were subcultured onto nutrient agar and identification was conducted using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Moore *et al.* 2016). In total, 243 isolates were identified using MALDI-TOF MS.

The measurements were performed with MALDI Microflex LT (Bruker Daltonics, Bremen, Germany). Protein profiles were acquired using linear-positive mode analysis with laser frequency at 20 Hz. Raw spectra were automatically acquired with AutoXecute control software (Flex control 3.4; Bruker Daltonics, Bremen, Germany) and were recorded within the range of 2,000 to 20,000 Da. The identification was performed using the MALDI BIOTYPER Software, version 3.1, with default parameters and the acquired spectra were compared with the mass-spectrum library (6.093 MSPs). The method was calibrated using the Bruker Bacterial Test Standard (BTS), a manufactured extract of *Escherichia coli* DH5 alpha spiked with two additional proteins (RNAase A and myoglobin) that extend the upper boundary of the mass range covered by BTS. Isolates identified as either *Elizabethkingia* spp. or *Chryseobacterium* spp. were kept frozen in glycerol stock solutions at −80°C until further use in the study.

The laboratory characteristics and biochemical profile of the isolates were examined after subculture on nutrient agar and 48 h incubation at 36°C.

To validate the initial identification of the strains by the standard MALDI Biotyper library, the 16S rRNA gene was amplified and sequenced. The bacterial isolates were cultured on nutrient agar and were incubated for 48 h at 36°C. Total DNA was extracted manually using the QIAamp DNA Mini kit (Qiagen). Total nucleic acid extracts (5 μL) were used for 16S rRNA gene amplification with Platinum *Taq* DNA Polymerase (Invitrogen) in a validated Eppendorf Mastercycler Gradient System. Master mixes (45 μL) with primer concentrations of 10 nmol/μL were prepared and amplified at 94°C for 5 min, 34 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by 72°C for 10 min. The primers used were 533F (5′-GTG CCA GCA GCC GCG GTA A-3′) and P1033R (5′-TGC GGG ACT TAA CCC AAC A-3′). Amplicons were separated, purified, and cycle sequenced with 533F and P1033R primers. Sequences were analyzed on ABI 3730XL genetic

analyzer (Applied Biosystems) and were compared to known gene sequences in GenBank and in BLASTSearch of MicrobeNet CDC Reference Laboratory. Furthermore, we submitted the acquired spectra to the mass spectrometry tool of MicrobeNet CDC Reference Laboratory using the Bruker-CDC merged library (7,737 MSPs).

Finally, the Bruker library was expanded by adding the standard mass spectra (MSPs) of the 16S rRNA sequenced strains to the existing database and an MSP dendrogram of the strains was constructed. Twenty-four raw spectra from the 16S rRNA sequenced strains were acquired using the protein extraction protocol according to the manufacturer's instructions. Each of the 24 raw spectra was submitted to baseline subtraction and smoothing procedure and afterwards processed using the MALDI Biotyper Offline Classification 3.1 software with the default parameters for MSP creation. The created MSPs were added to the existing database. The MSP dendrogram was constructed using the MALDI Biotyper Offline Classification 3.1 software with the default parameters for MSP dendrogram construction.

The antimicrobial susceptibility of the strains was tested by using MIC test strips (Liofilchem Diagnostics MIC Test Strip) following the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), version 7.1 (Breurec *et al.* 2016; Lau *et al.* 2016; Eriksen *et al.* 2017; Perrin *et al.* 2017).

RESULTS

Only three out of 243 isolates were identified as *Elizabethkingia* spp. by MALDI-TOF. This corresponds to three samples out of 457, collected from two hospitals out of eleven. The first isolate was classified as *E. meningoseptica* with a score value of 2.093, the second, originating from the same hospital was identified as *E. miricola* with a score value of 2.306, while the third strain was also identified as *E. meningoseptica* and demonstrated a score value of 2.394. The concentrations of the microorganism in the water samples were 2 cfu/100 mL, 16 cfu/100 mL, and 5,120 cfu/100 mL, respectively. Unfortunately, the two *Elizabethkingia* strains from the first hospital were not available for further analysis following their identification by MALDI BIOTYPER.

Subculture of the third strain onto nutrient agar gave white-yellow, shiny with entire edges colonies after 48 h of incubation at 36°C. No growth of the third strain on MacConkey agar was observed after 48 h incubation at 36°C. The results of the biochemical tests performed are demonstrated in Table 1.

16S rRNA gene sequence identified the isolate as *E. anophelis* (99% nucleotide identity to *E. anophelis* type strain R26, GenBank accession number MF615392 and 99.08% nucleotide identity with CSID_3015183678_outbreak strain after sequence analysis to CDC MicrobeNet BLASTSearch). *E. anophelis* is not included in the current Bruker reference library.

Submission of the peak list files of the isolate to the mass spectrometry tool of MicrobeNet CDC Reference Laboratory confirmed our results with identification score of the 16S rRNA sequenced strain 2.432 as *E. anophelis*. Using the protein profile of the 16S rRNA sequenced strain we expanded the custom MALDI-TOF database with the addition of *E. anophelis* MSP. Using the MALDI Biotyper Offline Classification 3.1 software, we repeated the identification of the saved protein profiles of the three isolates. It was shown that two of them were misidentified as *E. meningoseptica*, whereas the new classification with

Table 1 | Biochemical characteristics of *Elizabethkingia* isolate

Characteristics	*Elizabethkingia* strain
Indole production	+
H_2S production	−
Citrate utilization	−
Malonate utilization	−
Acid production from:	
Arabinose	−
Sucrose	−
Salicin	−
Lactose	+
Cellobiose	+
Mannitol	+
Trehalose	+
Growth on MacConkey agar	−
Hydrolysis of urea	−
ONPG	+

the expanded database correctly identified them as *E. anophelis* with score values of 2.358 and 2.816, respectively (Figure 1(a)). The *E. miricola* strain was correctly identified by MALDI-TOF. The MSP dendrogram of

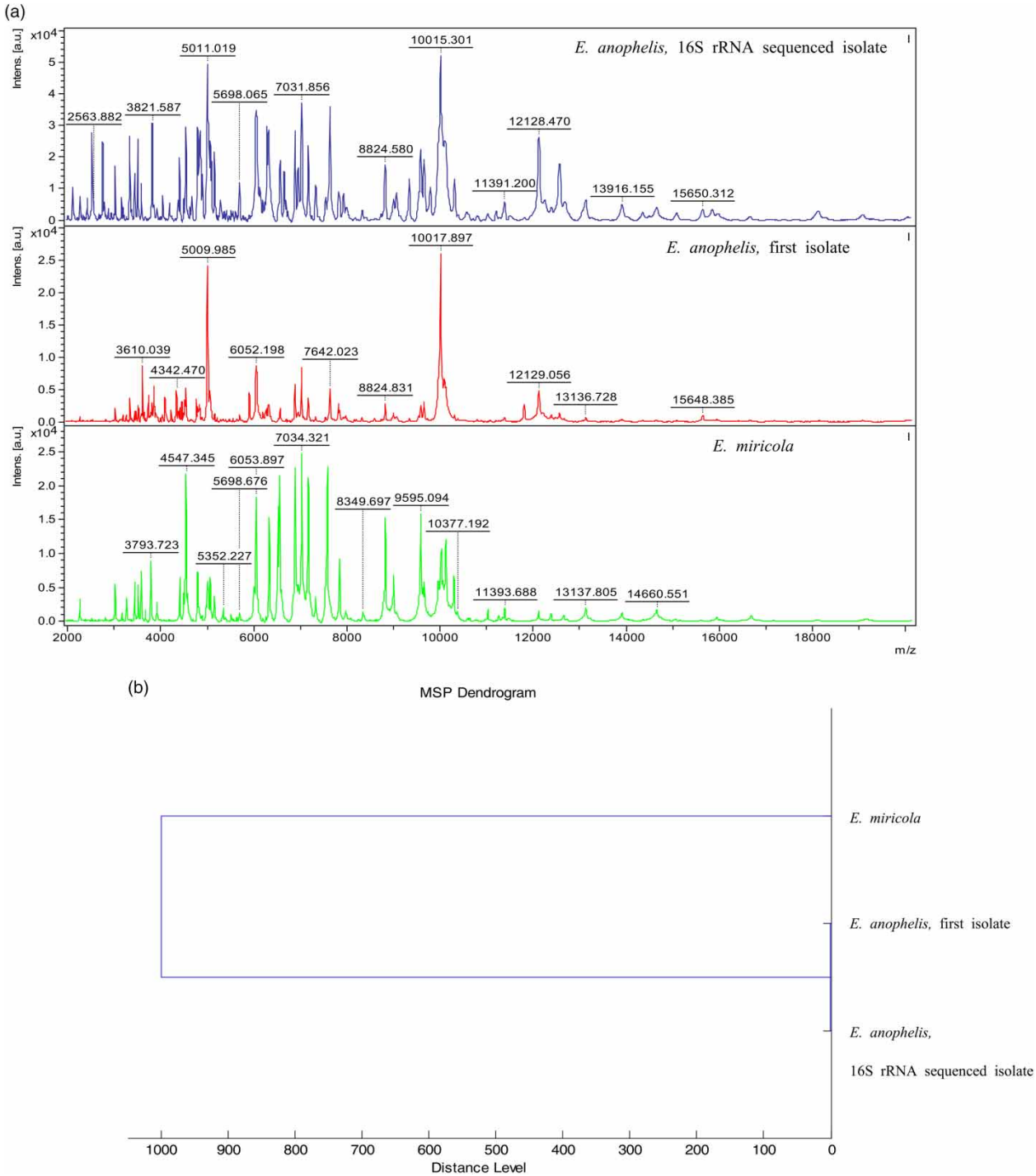

Figure 1 | Results of MALDI-TOF MS identification of the three *Elizabethkingia* isolates. (a) MALDI-TOF MS spectra of two *Elizabethkingia* species (*E. anopheles* and *E. miricola*) are shown. (b) Dendrogram generated from hierarchical clustering of MALDI-TOF MS spectra of the three *Elizabethkingia* isolates using ClinProTools 3.0 (Bruker Daltonics, Germany). Distances are displayed in relative units.

Table 2 | Antimicrobial susceptibilities of *Elizabethkingia* isolate determined by MIC strips

Antimicrobial agent	MIC (μg/mL)	Interpretation
Ciprofloxacin	0.25	S
Amicacin	>256	R
Amoxicillin	>256	R
Trimethoprim-sulfamethoxazole	0.25	S
Gentamicin	>256	R
Aztreonam	>64	R
Ceftazidime	>256	R
Tobramycin	>256	R
Amoxicillin-clavulanic acid	>256	R
Piperacillin-Tazobactam	8	S
Meropenem	>32	R
Imipenem	>32	R
Colistin	>256	R
Tigercyclin	0.25	S
Vancomycin	4	I

Submitted isolate to Genbank (accession no. MF615392). The results were interpreted according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing, version 7.1. for *Staphylococcus* spp. (vancomycin) and *Pseudomonas aeruginosa* (other antibiotics except for co-trimoxazole in which *Stenotrophomonas maltophilia* breakpoints were used), since there are no criteria for *Elizabethkingia*.

Elizabethkingia isolates clearly showed that there are two branches, one for *E. anophelis* strains and one for *E. miricola* (Figure 1(b)).

Antibiotic susceptibility testing of the *E. anophelis* strain showed that the strain was susceptible to ciprofloxacin, trimethoprime-sulfamethoxazole, piperacillin-tazobactam, and tigecycline, while resistant to amoxicillin, amoxicillin/clavulanic acid, ceftazidime, meropenem, imipenem, gentamicin, amikacin, tombamycin, and colistin. The isolate demonstrated intermediate *in vitro* susceptibility to vancomycin. The antibiotic susceptibility profile was consistent with the usual profile of the microorganism. Results are demonstrated in Table 2.

DISCUSSION

These are the first reported strains of *E. anophelis* isolated in hospital water systems, not only in Greece, but in Europe also, since despite our extensive research of the current literature, we did not find any other studies supporting the presence of the bacterium in hospital water supply systems. A probable explanation could be that *E. anophelis* is a new species, and isolates which were previously identified as *E. meningoseptica* could, in fact, be *E. anophelis* (Breurec *et al.* 2016; Lau *et al.* 2016; Janda & Lopez 2017). Although only one strain was used in our study in order to construct the *E. anophelis* MSP, the offline classification of the strains demonstrated satisfactory score values (2.358 and 2.816, respectively) and was in full concordance with the MicrobeNet CDC Mass Spectrometry classification. We will continue the research in order to enrich the reference database with more *E. anophelis* strains.

Even though our findings are consistent with those of researchers who suggested 16S rRNA gene sequencing as the gold standard method for the identification of *Elizabethkingia* spp. at the moment, since *E. anophelis* is not included in the Bruker MALDI-TOF reference library (Lau *et al.* 2015; Breurec *et al.* 2016; Eriksen *et al.* 2017), it should be taken into serious consideration that recent research indicates that *Elizabethkingia* spp. are very similar, when using 16S rRNA sequencing and there are still uncertainties regarding *Elizabethkingia* taxonomy (Doijad *et al.* 2016; Eriksen *et al.* 2017). Advanced molecular identification techniques are required for definite species identification (Janda & Lopez 2017). Nevertheless, the addition of *E. anophelis* to the MALDI-TOF MSP reference library will contribute to the rapid and reliable identification of this specific strain.

Recent studies have suggested that the hospital environment acts as a reservoir for the microorganism (Breurec *et al.* 2016), and that the microorganism persists in the hospital water microbiome despite the control measures (Moore *et al.* 2016). Although the strains detected three months apart in the water system of the same hospital belonged to two different *Elizabethkingia* species, our results could be considered supportive of previous findings.

In both hospitals, *Elizabethkingia* spp. infections were not recorded, but two cases of *Elizabethkingia*-like species (*Chryseobacterium indologenes*) infections were recorded in the first hospital during 2016. One possible reason could be that in Greece, *Elizabethkingia* infections are probably underdiagnosed, since automated systems or biochemical tests, which are the most prevalent used methods for identification in Greek hospitals, may misidentify the microorganism (Centers for Disease Control and Prevention

2016). Further studies for the virulence of the strains isolated should be performed since strains that could lead to an outbreak seem to have several characteristic pathogenic genomic regions (Perrin *et al.* 2017). Since there are no recorded cases of *Elizabethkingia* infections in Greece, the Greek medical community should be aware about the clinical significance of the bacterium in order to be alert.

CONCLUSIONS

Given the severity of the infection in hospitalized patients (Lau *et al.* 2016), the multidrug-resistant profile (Lau *et al.* 2016), the persistence of the microorganism in the environment (Breurec *et al.* 2016), and the unknown way of transmission and pathogenesis, we should consider that *E. anophelis* may be a cause of nosocomial infection in Greece. In that respect, infectious diseases specialists, microbiologists, and epidemiologists should actively search for the pathogen both in clinical and environmental samples.

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