Corynebacterium sp. isolated from blood culture of a bacteremic patient. Will the assumptions about a new corynebacterium be confirmed?

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ABSTRACT

A case report is presented of a patient with suspected septicaemia from whose blood culture a new strain of *Corynebacterium* sp. was isolated. Until now, no report of this strain isolated from human clinical materials has been available in the literature. In addition to a brief clinical description of the case, the article also features morphological, biochemical properties as well as antibiogram of the bacterium. It describes also methods used for the identification of this isolate. The aim of the work was to highlight a novel and rare coryneform strain.

KEYWORDS

Corynebacterium sp. – septicaemia – blood culture – WGS

SOUHRN

Mališová L., Ježek P., Dresler J., Chmel M., Španělová P., Musílek M., Šafránková R., Žemličková H.: *Corynebacterium* sp. izolovaný z hemokultury bakteriemického pacienta. Potvrdí se předpoklady o novém korynebakteriu?

Kazuistika suspektní septikemie s izolací raritního kmene *Corynebacterium* sp. Izolace tohoto kmene ani kmenů geneticky blízkých druhů z klinického materiálu nebyla zatím v dostupné literatuře popsána. V článku jsou uvedeny kromě stručného klinického popisu také morfologické a biochemické vlastnosti, metody identifikace a vyšetření k antibiotikům. Cílem práce bylo především upozornit na nový a vzácně se vyskytující kmen korynebakteria.

KLÍČOVÁ SLOVA

Corynebacterium sp. – septokemie – hemokultura – WGS

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INTRODUCTION

In microbiological practice, non-diphtheria corynebacteria have been historically considered as contaminants or saprophytic flora of the skin and mucous membranes of humans. On the other hand, it is known that immunosuppressed patients and implant recipients are particularly at risk of infection caused especially by corynebacteria species. In the last decades, the genus *Corynebacterium* has experienced considerable changes in terms of the number of newly described species and their clinical presentation. Based on recent literature data, the genus comprises more than 90 species that are found in milk products, plant leaves, sewage, soil, and in the aquatic environment [1]. Some species were also isolated from animals and humans. *Corynebacterium* species may differ in their pathogenicity, ranging from opportunistic

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(*C. amycolatum*, *C. striatum*, *C. jeikeium*, *C. macginleyi* etc.) to overt pathogens (C. *diphteriae, C. ulcerans)*. Some species were isolated frequently or exclusively from blood cultures. There were isolated eight *Corynebacterium* species from blood cultures, including those that have not yet been isolated from sites other than the bloodstream or are the most common found in the bloodstream in the Příbram (Figure 1) [10].

The genus *Corynebacterium* is highly heterogeneous. The bacteria are straight or slightly curved Gram-positive rods, with a tendency to form typical V-shaped arrangements (with acute angle) and club-shaped swellings. Sometimes, metachromatic granula are found inside the cells. They show good aerobic or facultative anaerobic growth, are asporogenous and non-acid resistant. Based on metabolic properties, corynebacteria can be assigned to different groups. The species requiring a lipid component to grow on culture media are termed lipophilic (e. g. *C. jeikeium, C. ureolyticum, C. accolens, C. macginleyi*, etc.) while those without these needs are termed non-lipophilic. They can be further classified according to their metabolic activity into sucrose fermenting (e. g. *C. diphtheriae, C. xerosis, C. amycolatum,* etc.) and nonfermenting (e. g. *C. pseudodiphtheriticum, C. afermentans ssp. afermentans,* etc.) [4].

In the last decades, considerable attention has been devoted to this genus. Multiple novel *Corynebacterium* species and number of case reports with a suspected *Corynebacterium* etiology have been published [5, 6]. The structure and characteristics of the hospitalized patients have also changed. The number of patients with different levels of immunosuppression and of those who underwent intensive and/or invasive therapy is ever increasing, and such patients provide niches for opportunistic pathogens. Moreover, modern methods of identification of microorganisms are used.

Case Report

A 44-year-old man with schizophrenia who had threatened to commit suicide by jumping out of the window and then was admitted to intensive care unit (ICU) of the Regional Hospital Příbram (Czech Republic). He was transported to the hospital by the emergency ambulance service after experiencing cerebral hypoxia for approximately 10 minutes. After successful resuscitation, the patient remained quadriplegic with no spontaneous or induced motor activity of the extremities. A central venous catheter was inserted, tracheostomy was done, and full mechanical ventilation was provided.

A tracheal aspirate (TAS) was collected on the day after hospital admission, and based on microscopy, high numbers (10^7/ml) of pneumococcal-like cells were suspected. According to the presumptive identification and clinical symptoms, the treatment with benzylpenicillin (5 MIU four times a day) was iniciated. During the

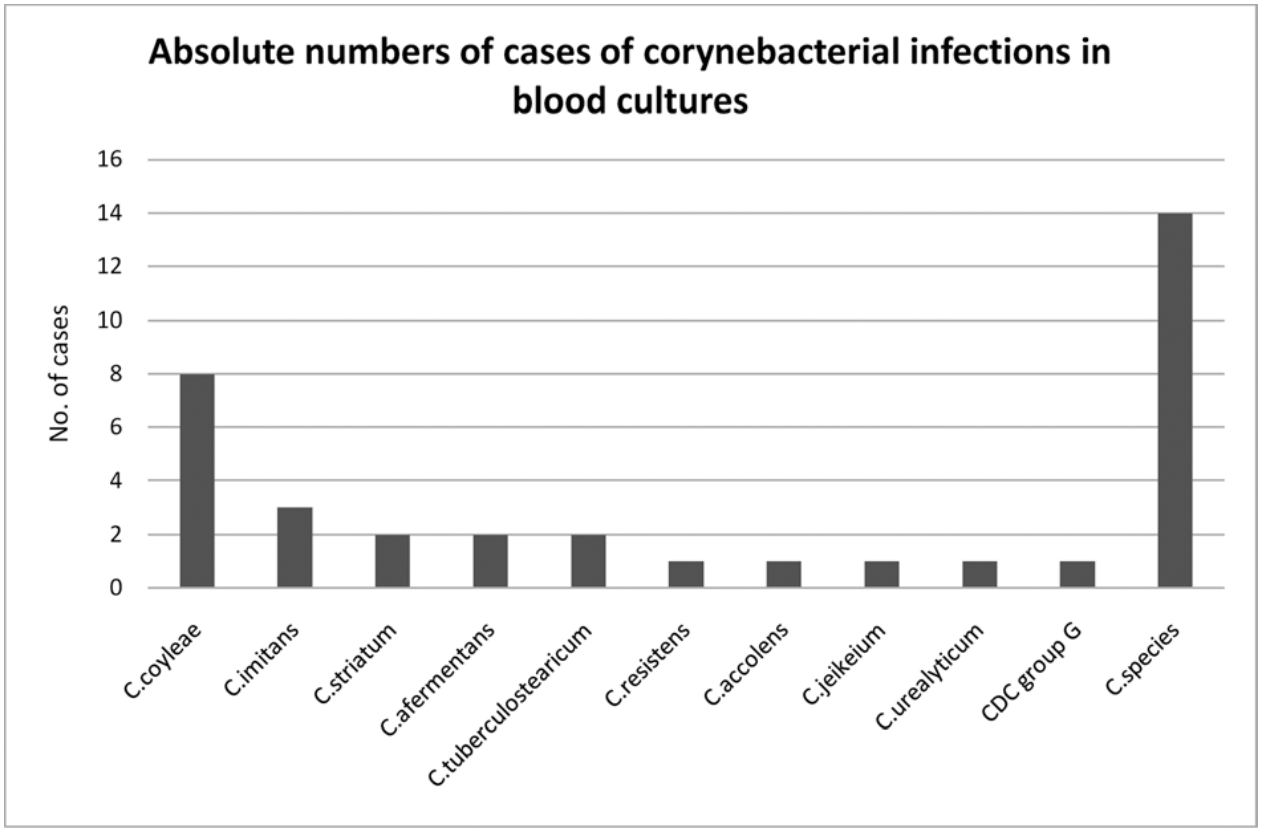

Figure 1. Corynebacterial bacteremia in the Regional Hospital Pribram (Czech) in the years 2012–2016 [10]

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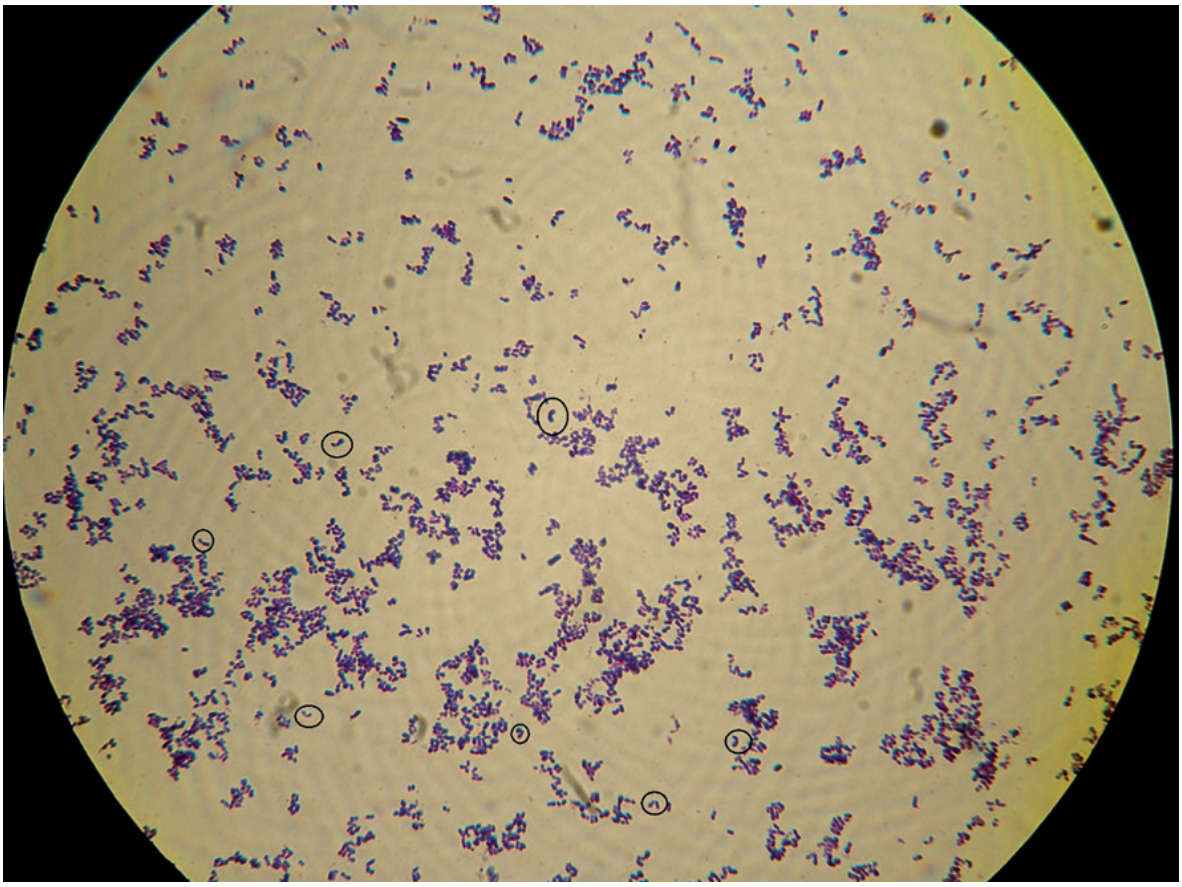

Figure 2. Morphological visualisation of *Corynebacterium sp.* after Gram stain
Typical V-shaped forms are highlighted in circles

therapy, CRP rose from 5.4 mg/L at admission progressively to 250 mg/L on the following days, with mild leukocytosis (12.9 x 10^9/L). However, a pneumococcal etiology was not confirmed by culture. As clusters of Gram-positive cocci were observed on microscopy of the positive blood culture, the patient was switched to high-dose oxacillin (4 x 3 g) on day 3 of treatment with benzylpenicillin. The patient became responsive to the current treatment and his condition was progressively improving. His inflammatory markers decreased and when transferred to the post-intensive care on day 13 after admission, leukocytes became normal and CRP dropped to the value of 43.6 mg/L.

In meantime, the strain isolated from blood culture was referred to the Czech National Collection of Type Cultures (CNCTC) at the National Institute of Public Health (Prague, Czech Republic) for identification. The strain was deposited as CNCTC 7651. While in hospital, the patient underwent microbiological testing of TAS (several times), endotracheal (ET) cannula contents, several sets of blood culture and bronchoalveolar lavage (BAL) fluid (once). Apart from *Haemophilus influenzae* whose clinical relevance was unlikely and was detected from TAS and ET cannula contents but not from BAL fluid throughout the hospital stay, no other suspected pathogen was recovered.

Neither was isolated a microorganism comparable to the strain from blood culture in any of the collected specimens referred for microbiological analysis. Its origin thus remained unclear.

Due to the slow growth of the isolate, it was investigated only by microscopy and corynebacterial cells has been observed (Figure 2).

MICROBIOLOGY

Corynebacterium was isolated from one of the three collected haemocultures taken at the time of patient admission and showed very slow growth under aerobic, microaerophilic, and anaerobic conditions. No marked difference in the growth was found between different culture conditions. After 24 hours of culture, only negligible growth was observed, and after 48 hours of culture at 37 °C, the strain grew in tiny, dry, greyish colonies without evident hemolysis. In a microscopic mount from the culture, the morphology of tiny coryneform rods was visible. This tiny cell morphology could have been the cause of the misidentification of the microscopically positive culture as staphylococci or pneumococcal cells on microscopy of tracheal aspirate. Nevertheless, cells from further subculture already had

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typical *Corynebacterium* characteristics (typically V and club shaped) – Figure 2. In API®Coryne (BioMerieux, France), after 48 hours at 37 °C, pyrazinamidase and alkaline phosphatase activity was recorded (Table 1). The study strain showed weak acid production from glucose. Even after 96 hours of incubation, this production was very mild. With the profile number 2100104, an identification score of 80%, and a T index of 1.0, the identification pointed to *Corynebacterium argentoratense*. Nevertheless, the strain can be differentiated from *C. argentoratense* still on the plate as *C. argentoratense* does not require a lipid component to grow and grows well on Columbia agar as early as within 24 hours, in colonies that are typical for non-lipophilic species. Based on ambiguous results of biochemical testing, a further examination was provided for precise identification of the strain. All reactions of API®ZYM (BioMerieux, France) corresponded to the type strain of *C. aquatimens* [1]. Additional tests, the CAMP test was negative, the catalase test was positive, and the oxidase test was negative. MALDI-TOF mass spectrophotometry (MALDI-TOF, Microflex Brucker; Bremen, Germany) was not able to identify the strain what can be explained by the absence of *C. aquatimens* in its database. Therefore, a sequencing of 16S rDNA was used for identification of mentioned isolate. DNA of the strain was isolated according to the manufacturer's protocol using a commercial kit (Bio Basic Canada Inc., BS 624) and sequenced with set of primers [8] using Sanger sequencing (Applied Biosystems 3130xL). An approximately 1200bp long sequence was analysed by Bionumerics 7.6.2 (Applied Maths Ghent, East Flanders Belgium). The analysed isolate shared 98% similarity with the type strain of *C. aquatimens* (IMMIB L-2475, CNCTC 8065) [1]. Whole genome sequencing was performed for both the analysed isolate and the type strain of *C. aquatimens*. Genomes were sequenced on Oxford Nanopore (ONT GridIONx5 platform, R9.4.1 chemistry (Flow Cell), Ligation Sequencing Kit (SQK-LSK108)) and Illumina (iSeq 100 Sequencing System, the library was prepared with Nextera XT DNA Library Preparation Kit), assembled by CANU [7] and polished with Pilon [11]. Sequence of CNCTC 7651 was uploaded to genBank and it is available under accession number CP071246. Average nucleotide identity (ANIb) between the two genomes calculated with JSpecies (http://jspecies.ribohost.com/jspeciesws/) was 73%, distinctively below the cutoff (95%). Genetic relations of the genomes were displayed in the phylogenetic tree constructed from the collection consisting of mentioned genomes, genomes of medically relevant species [2], *C. glaucum* and *C. aquatimens*. Phylogeny based on single copy orthologs determined by OrthoFinder [3] was constructed using RaxML [9]. Resulting tree (Figure 3) was differentiated into four clades and several separate branches. The genomes

Table 1. Biochemical properties of *Corynebacterium* sp.

1. Test API Coryne®		2. Test API ZYM®		3. Test API Coryne® API ZYM®	
ESC	-	EST	+	PAL	+
URE	-	ESL	+	βGUR	-
GEL	-	LIP	-	βGAL	-
GLU	+	LEU	+	αGLU	-
RIB	-	VAL	-	NAG	-
XYL	-	CYS	-		
MAN	-	TRY	-	4. Additional biochemical testing	
LAC	-	αCHY	-		
SAC	-	PAC	+	CAMP	-
GLYG	-	NPH	-	CAT	+
NIT	-	αGAL	-	MAL	-
PYZ	+	βGLU	-	OXI	-
PyrA	-	αMAN	-	VPT	+
		αFUC	-		

API Coryne®, API ZYM® and additional biochemical testing

ESC – esculin; URE – urease; GEL – gelatine hydrolysis; GLU – fermentation of glucose; RIB – fermentation of ribose; XYL – fermentation of xylose ; MAN – fermentation of mannitol; LAC – fermentation of lactose; SAC – fermentation of sucrose; GLYG – fermentation of glycogen; NIT – reduction of nitrates; PYZ – parazinamidase; PyrA - pyrrolidonyl arylamidase; EST – esterase (C4); ESL – esterase lipase (C8); LIP - lipase (C14); LEU – leucine arylamidase; VAL – valin arylamidase; CYS – cysteine arylamidase; TRY – trypsin; αCHY – α chymotrypsin; PAC – acid phosphatase; NPH – naphtol-AS-BI-phosphohydrolase; αGAL – α galactosidase; βGLU – β glucosidase; αMAN - α mannosidase; αFUC – α fucosidase; PAL – alkaline phosphatase; βGUR – β glucuronidase; βGAL – β galactosidase; αGLU – α glucosidase; NAG – N-acetyl-β-glucosaminidase; CAMP – CAMP reaction; CAT – production of catalase; MAL – fermentation of maltose; OXI – oxidase; VPT – Voges-Proskauer test

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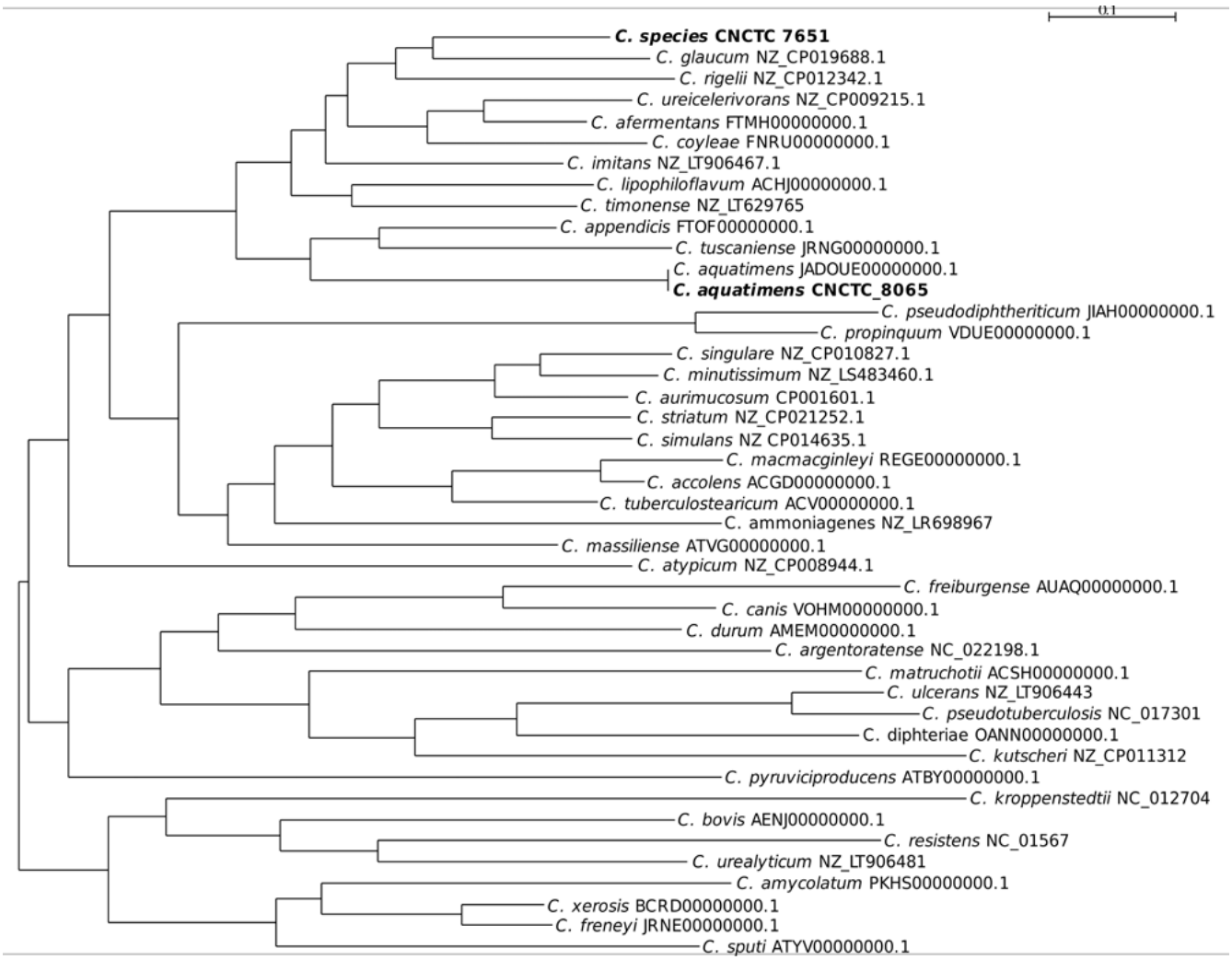

Figure 3. Phylogenetic tree reconstructed of medically relevant species of genus *Corynebacterium, C. glaucum, C. aquatimens,* the strain CNCTC 7651 and the strain 8065

Table 2. Antibiotic susceptibility

Antibiotic	Inhibition zone (mm)	MIC (mg/L)	Antibiotic	Inhibition zone (mm)	MIC (mg/L)
Penicillin	**26***	**0.125***	Linezolid	**47**	**0.25**
Gentamicin	**26**	**0.025**	Rifampicin	**43**	**0.008**
Ciprofloxacin	**28**	**0.25**	Clindamycin	**31**	**0.032**
Tetracycline	**30**	**0.25**	Vancomycin	**22**	**0.25**

**EUCAST Clinical Breakpoints v.11.0 are 29 mm for DDST and 0.125 for MIC*

of *C. glaucum*, *C. aquatimens and* CNCTC 7651 were positioned in the same clade, the nearest neighbour of CNCTC 7651 was *C. glaucum,* nevertheless, genetic distances confirmed that these belong to different species.

The susceptibility to antibiotics was tested by the disk diffusion method and broth microdilution method according to the recommendation of European Committee on Antimicrobial Susceptibility Testing (EUCAST), version 11.0 (http://www.eucast.org). Based on the results, the isolate turned out to be susceptible to almost all tested antibiotics. Data are given in Table 2.

DISCUSSION

In recent years, a number of novel *Corynebacterium* species originated from human and animal clinical specimens have been described. This is also the case with C*orynebacterium sp.*. described in this publication (man, 44y, strain isolated from blood). In our study, the detection of the bacterium was linked to elevated inflammatory markers (mainly CRP and initially also leukocytosis). A question arises as to whether the outcome was a result of the antibiotic therapy or whether bacteraemia cleared spontaneously. Penicillin which showed

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questionable *in vitro* efficacy was given only initially and after that, the therapy was switched to oxacillin, whose therapeutic effectiveness cannot be assessed because breakpoints are not establish and susceptibility cannot be evaluated. In other clinical specimens collected during the patient's hospital stay, no such strain has been isolated and the focus of septicaemia remains unknown. Even contamination during the collection of the sample cannot be ruled out although this species has not yet been reported as resident flora of the skin or mucous membranes of humans or animals. Moreover, the possibility of inaccurate microbiological identification of bacterial cells acquired from tracheal aspirate and hemoculture cannot be exclude.

From the description provided above, it is apparently that due to lack of compelling evidence, the detection of *Corynebacterium sp.* from blood culture cannot be considered as linked to the clinical condition. However, it was not the purpose of the publication to evaluate the clinical context.

Sequencing analysis of this isolate revealed the presence of *Corynebacterium* spp. in the clinical specimen. Considering to the lower discriminatory power of 16S rDNA compared to whole genome sequencing analysis, it seems that this method is not sufficient for discrimination of the genus *Corynebacterium* to species level due to genetic relationships within the genus therefore, the isolate was originally identified incorrectly. Phylogenetic analysis confirmed that CNCTC 7651 is on subgenus level genetically related to several medically relevant *Corynebacterium* species isolated from blood as well as to *C. aquatimens* and *C. glaucum*.

CONCLUSION

We present a case report of a patient with suspected septicaemia with no clear clinical link to the agent isolated, and highly probably the first report of a new corynebacterial species. The properties and antimicrobial susceptibility of the study strain are also presented. To determine its involvement, if any, in some human diseases, further experience and reports would be needed, as is the case with other rare detections of the *Corynebacterium* genus. The aim of the communication was not to demonstrate the clinical relevance. The aim of this study was to identify rare occurring bacteria isolated from blood using biochemical testing and molecular biology method and to point out the possible occurrence of a new species of corynebacteria.

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