

Characterization and antimicrobial susceptibility of one antibiotic-sensitive and one multidrug-resistant *Corynebacterium kroppenstedtii* strain isolated from patients with granulomatous mastitis

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Abstract

Human infections associated with *Corynebacterium kroppenstedtii* are rarely reported, and this organism is usually described as antibiotic sensitive. Almost all published cases of *C. kroppenstedtii* infections have been associated with breast pathology in women and have been described in New Zealand, France, Canada, India and Japan. Here we describe the microbiologic characteristics of two strains isolated from two women diagnosed of granulomatous mastitis in Spain. One *C. kroppenstedtii* isolate was antibiotic sensitive while the other was multidrug resistant. Biochemical identification was possible using a wide battery of methods including API Coryne V2.0, API Strep, API NH, API NE, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and 16S rRNA gene amplification and sequencing. Antimicrobial susceptibility to 28 antibiotics as determined by Etest showed one isolate being sensitive to benzylpenicillin, ciprofloxacin, moxifloxacin, gentamicin, vancomycin, clindamycin, tetracycline, linezolid and rifampin. The second isolate showed resistance to ciprofloxacin, moxifloxacin, clindamycin, tetracycline and rifampin. The multidrug-resistant isolate contained the *erm*(X), *tet*(V), *cmx*, *aphA1-IAB*, *strAB* and *sulI* resistance genes known from the R plasmid pJA144188 of *Corynebacterium resistens*. These genes were absent in the genome of the antibiotic-sensitive isolate. This report confirms the tropism of this microorganism for women's breasts and presents the first description of a multidrug-resistant *C. kroppenstedtii* strain.

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Introduction

Corynebacterium kroppenstedtii was first described in 1998 as a lipophilic species of the genus *Corynebacterium* lacking the

characteristic mycolic acids of the corynebacterial cell envelope [1]. The description of this new species was based on one strain isolated from the sputum of an 82-year-old woman with chest infection and was deposited in the Culture Collection of the University of Göteborg (CCUG, Sweden). Genome sequencing of the type strain *C. kroppenstedtii* CCUG 35717 (DSM 44385) revealed a contiguous genomic sequence with a total size of 2 446 804 bp and 2122 protein-coding regions [2]. The lack of corynemycolic acids and the lipophilic lifestyle of *C. kroppenstedtii* are caused by several events of gene loss, including a condensate gene cluster and a mycolate reductase gene, both involved in mycolic acid biosynthesis and a microbial type I fatty acid synthase gene, resulting in a fatty acid

auxotrophy [2]. According to metadata from the Human Microbiome Project, *C. kroppenstedtii* is an abundant species in the retroauricular crease [3], and it preferentially colonizes the anterior nares and other distinct skin sites [4].

Since the description of the clinical isolate from Sweden, which was used to characterize this new corynebacterial species taxonomically, additional isolates of this organism from human clinical sources have been reported in New Zealand [5,6], France [7–10], Canada [11], India [12], Japan [13–15] and Germany [16]. Eighty-eight percent of isolates have been obtained from women's breasts; the rest of the isolates came from sputum [1,11], lung biopsy sample [11], blood [11] and endocardiac valve [16]. From animal sources, a case of external otitis associated with this organism was reported in a peach-faced lovebird [17]. A comprehensive microbiologic and clinical review of this organism has been published recently [18].

The aims of this work were to present the microbiologic profile of two *C. kroppenstedtii* strains, including their identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequencing, and to provide information on their antimicrobial susceptibility to 28 antibiotics. This analysis led to the first detection of a multidrug-resistant *C. kroppenstedtii* strain.

Material and Methods

Strains characterized in this study

Two coryneform strains (CNM632/14 and CNM633/14) were isolated in pure culture from clinical samples obtained by fine needle aspiration from the breast of two women, 38 and 45 years old, diagnosed with granulomatous mastitis and treated at the Complejo Asistencial Universitario de León, Spain.

Phenotypic identification of CNM632/14 and CNM633/14

API Coryne V2.0 reactions (bioMérieux, Marcy l'Etoile, France), API Strep (hippurate hydrolysis), API NH (fructose fermentation) and API NE (assimilation of maltose, *N*-acetyl-glucosamine and phenylacetic acid) were used for the phenotypic characterization of the corynebacterial isolates. In addition, catalase and oxidase activity, lipophilia, Christie–Atkins–Munch–Peterson (CAMP) reaction, glucose fermentation at 42°C, growth on blood agar at 20°C and susceptibility to vibriostatic factor O/129 were examined following previously described methods [19]. MALDI-TOF MS was carried out with a Bruker Biotyper system (Bruker Daltonics, Bremen, Germany). Software version MBT 3.1 and the BDAL 5627 library were used for the bacterial identification. The direct colony method including the spotting onto a MALDI-TOF MS target plate

covered with 1 µL of formic acid (100%) and 1 µL of matrix was used as previously described [20]. Scores of ≥ 1.5 and ≥ 1.7 were used for genus and species identification, respectively [21].

Genotypic identification and 16S rRNA gene sequences

Amplification of the 16S rRNA genes and sequencing of the PCR products were performed according to previously described methods [22]. The 16S rRNA gene sequences of the studied strains CNM632/14 and CNM633/14 were assigned the GenBank accession numbers KP230546 and KP230545.

Antimicrobial susceptibility testing according to EUCAST criteria

Antimicrobial susceptibility testing to 28 antimicrobials was determined by Etest on Mueller-Hinton agar with 5% sheep's blood, incubated in air at 35°C and read after 48 hours. Susceptibility to antibiotics was interpreted following recommended criteria by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for coryneform organisms (<http://www.eucast.org/>).

Results

Isolation of *C. kroppenstedtii*

Two coryneform strains, CNM632/14 and CNM633/14, were obtained from samples taken from women's breasts in pure culture and grown on blood agar in air at 35°C for 48 hours. Colonies were less than 1 mm in diameter, circular, smooth, matte, nonpigmented and nonhaemolytic. The bacterial growth was enhanced on blood agar supplemented with 1% (v/v) Tween 80.

Species identification by microbiologic and molecular methods

Both strains presented the same API profiles after 24 hours (2100104, maltose, sucrose and aesculin negative) and after 48 hours (2140104, maltose and sucrose negative, aesculin positive), suggesting *Corynebacterium argentoratense* and *Corynebacterium jeikeium*, respectively. Positive reaction was observed for catalase, hippurate hydrolysis and pyrazinamidase and weak reaction for alkaline phosphatase. Aesculin was weakly hydrolyzed after 48 hours. Acid was produced from glucose and fructose but not from maltose, sucrose, ribose, xylose, lactose, mannitol and glycogen. The microorganisms were negative for oxidase, urease and nitrate reductase. Negative reactions were also observed for gelatine hydrolysis, β -glucuronidase, β -galactosidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, CAMP test and the assimilation of maltose, *N*-acetyl-

glucosamine and phenylacetic acid. Both isolates were inhibited by the O/129 factor (150 µg), showing an inhibition diameter of 30 and 42 mm, respectively.

Using MALDI-TOF MS as previously described, the two coryneform strains gave significant scores for *C. kroppenstedtii* of 1.990 and 1.780 (data not shown). The sequenced DNA fragments of the 16S rRNA gene were 1328 bp (CNM632/14) and 1146 bp (CNM633/14) in length. The nucleotide sequence similarities with GenBank sequences of *C. kroppenstedtii* were 99.4 and 100% to *C. kroppenstedtii* CIBU 090024 (JF299190) from France [9], 99.1 and 99.7% to *C. kroppenstedtii* 00-0244 (AF537598) from Canada [11] and 99.0 and 99.7% to the type strain *C. kroppenstedtii* DSM 44385 (CP001620) [2]. These data clearly demonstrated that both clinical isolates obtained from granulomatous mastitis can be assigned to the species *C. kroppenstedtii*.

Antimicrobial susceptibility profiling of the new *C. kroppenstedtii* isolates

The antimicrobial susceptibility data for the two *C. kroppenstedtii* isolates tested against 28 antimicrobials are presented in Table 1. Strain 1 (CNM632/14) showed, with the exception of cefixime, very low minimum inhibitory concentration (MIC) values for the rest of the antibiotics tested. However, the second strain (CNM633/14) showed high MIC values for many antibiotics tested including cefixime, erythromycin, clarithromycin, azithromycin, clindamycin, quinupristin–dalfopristin, tetracycline, chloramphenicol, ciprofloxacin, levofloxacin, streptomycin, kanamycin and cotrimoxazole. Applying the EUCAST recommendations for antimicrobial susceptibility, the first isolate can be considered sensitive to benzylpenicillin, ciprofloxacin, moxifloxacin, gentamicin, vancomycin, clindamycin, tetracycline, linezolid and rifampin. The second isolate showed resistance to ciprofloxacin, moxifloxacin, clindamycin, tetracycline and rifampin.

Discussion

Granulomatous mastitis is an inflammatory breast disease of unknown aetiology that generally affects women of child-bearing age, usually within a few years after they have given birth [5,6]. The disease can become chronic and disfiguring, and it can be confused with a tumour. Mammary granulomas have been associated with tuberculosis, sarcoidosis, fungal infections and Wegener granulomatosis, but since 2002 it has also been associated with infection by *C. kroppenstedtii* [5]. Our strains were recovered from two patients with granulomatous mastitis, representing the first reported cases in Europe outside of France. The breast is rich in lipids—a favourable condition

TABLE 1. Antimicrobial susceptibility of two *Corynebacterium kroppenstedtii* strains to 28 antimicrobials

Antimicrobial	Strain 1 (CNM632/14)		Strain 2 (CNM633/14)	
	MIC (mg/L)	Category ^a	MIC (mg/L)	Category ^a
Benzylpenicillin	0.064	S	0.023	S
Ampicillin	0.047	NA	<0.016	NA
Cefuroxime	0.75	NA	1	NA
Cefixime	>256	NA	>256	NA
Cefotaxime	0.047	NA	0.023	NA
Imipenem	0.016	NA	0.047	NA
Vancomycin	0.75	S	0.38	S
Teicoplanin	1	NA	1	NA
Linezolid	0.064	S	0.064	S
Daptomycin	0.094	NA	0.25	NA
Tetracycline	1	S	32	R
Tigecycline	0.032	NA	0.064	NA
Chloramphenicol	0.75	NA	>256	NA
Ciprofloxacin	0.094	S	>32	R
Moxifloxacin	0.064	S	1.5	R
Levofloxacin	0.094	NA	>32	NA
Erythromycin	<0.016	NA	>256	NA
Clarithromycin	<0.016	NA	>256	NA
Azithromycin	0.023	NA	>256	NA
Clindamycin	0.032	S	>256	R
Quinupristin–dalfopristin	0.38	NA	>32	NA
Streptomycin	0.75	NA	12	NA
Kanamycin	0.25	NA	>256	NA
Gentamicin	0.016	S	0.032	S
Tobramycin	0.023	NA	0.032	NA
Amikacin	0.125	NA	0.125	NA
Rifampin	<0.002	S	<0.002	S
Cotrimoxazole	0.032	NA	>32	NA

MIC, minimum inhibitory concentration; NA, not available; R, resistant; S, susceptible.
^aAccording to European Committee on Antimicrobial Susceptibility Testing (EUCAST) susceptibility breakpoints (<http://www.eucast.org/>).

for the development of lipophilic corynebacteria. Moreover, in histologic sections of breast samples, the organism has been located in vacuoles that probably contained lipids [5]. These findings coincide with the evaluation of the genomic data of *C. kroppenstedtii* which indicate that lipophilism (i.e. the dependency of bacterial growth on the presence of lipids) is the dominant feature involved in pathogenicity of *C. kroppenstedtii* [2]. The role of this organism as a cause or complicating factor of granulomatous mastitis has been demonstrated by several authors [18], but such a disease seems to be a complex entity in which the presence of *C. kroppenstedtii* could be just one piece of an as-yet unsolved puzzle [18]. Further clinical and histopathologic studies are still necessary to clarify its real role in such a disease.

The new *C. kroppenstedtii* isolates showed the typical phenotypic characteristics previously described for this microorganism [18]. Identification of this bacterium was achieved by phenotypic methods including the API Coryne obtaining profiles of 2100104 and 2140104 after 24 and 48 hours respectively, with additional tests needed for a full phenotypic identification. Results of biochemical tests with lipophilic corynebacteria should be interpreted after at least 48 hours of incubation, as acid production from carbohydrates and aesculin hydrolysis might be delayed in many of these organisms

[18,23]. Using MALDI-TOF MS as previously described, our two strains gave scores of ≥ 1780 , which is considered reliable to accurately identify the species [20,21]. Definitive identification was also obtained by sequencing the 16S rRNA gene [18,22], and our two strains presented nucleotide sequence similarities with GenBank sequences of *C. kroppenstedtii* from 99 to 100%. The species identification was furthermore corroborated by genome sequencing and the deduced genomic similarity of both strains to the type strain *C. kroppenstedtii* DSM 44385 [2,24].

Using the Etest method and applying the EUCAST recommendations for antimicrobial susceptibility of coryneform organisms (<http://www.eucast.org/>), the two *C. kroppenstedtii* strains were found to be uniformly sensitive to penicillin, vancomycin, linezolid, gentamicin and rifampin. One isolate was resistant to tetracycline, ciprofloxacin, moxifloxacin and clindamycin. It is striking that EUCAST, unlike the recommendations established by the Clinical and Laboratory Standards Institute [25], has not yet defined breakpoints for susceptibility to erythromycin, although high MIC values are usually reported for corynebacteria, and even genes coding for macrolide resistance are often found [18].

The phenotypical novel multidrug resistance of *C. kroppenstedtii* CNM633/14 was confirmed when the genome sequence was analysed and several antibiotic resistance genes were annotated [24]. Interestingly, most of them are located in a specific genomic island with similarity to the R plasmid pJA144188, conferring multidrug resistance to *C. resistens* DSM 45100 [26]. In addition, the genomic island includes all insertion sequences previously described in pJA144188. The gene content of the resistance island is fully consistent with the antimicrobial susceptibility data (Table 1), as *erm(X)*, *tet(W)*, *cmx*, *aphA1-IAB*, *strAB* and *sulI* can confer resistances to MLSb antibiotics, tetracyclines, chloramphenicol, aminoglycosides, streptomycin and sulfonamides in corynebacteria [24].

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Conflict of Interest

None declared.

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