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Comparative analysis of different phenotypic and molecular methods used for the taxonomic identification of *Corynebacterium spp*. isolated from clinical samples in Romania

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Abstract

The infections produced by non-diphtheriae Corynebacterium spp. in Romanian patients are poorly documented and probably neglected. The aim of our study is to characterize Corynebacterium strains collected from Romanian hospitalized individuals and outpatients in order to be identified by phenotypic and molecular methods.Twenty Corynebacterium spp. isolates were identified at the species level using phenotypic and molecular techniques. Phenotypic identification consisted in standard microbiological methods: Gram stain, catalase and oxidase tests and biochemical characterization using the commercial API Coryne kit according to the manufacturer's instructions and Maldi-toff method. The final identification of species was achieved by molecular technique, namely ribosomal DNA sequence analysis. The strains recovered from human clinical samples (six from blood culture, five isolates from wounds, two from peritoneal fluid, three from urine, one from a drainage catheter, one from perirectal abscess, one from osteomyelitis infection and one from conjunctival secretions) were identified as Corynebacterium striatum (n=7), C. amycolatum (n=7), C. urealyticum (n=3), C. afermentans (n=2) and one isolate was classified as C. pseudodiphthericum.

Keywords*:* Key words: *Corynebacterium* spp., phenotypic methods, molecular technique, 16S ribosomal DNA identification

1. Introduction

Genus *Corynebacterium* includes Gram positive, pleomorphic, catalase positive, immobile, non-capsulated, non-sporulated and non-acid-fast bacilli. The genus is currently composed of more than 120 species and subspecies isolated from human, animal and environmental sources [1]. The genus includes three potential toxigenic species: *C. diphtheriae* with 4 biotypes (gravis, mitis, intermedius and belfanti), *C. ulcerans* and *C. pseudotuberculosis*. A great number of other corynebacteria are members of the normal skin and the upper respiratory tract microbiota. Many of these can cause infections, especially in immunocompromised or debilitated human patients (G. Funke et al. [2], L. Martínez-Martínez

[3]). *Corynebacterium xerosis, C. amycolatum* and *C. striatum*, are the most frequently isolated from clinical samples (F.N.R. Renaud et al. [4]). In 1988, Collins and associates isolated a new *Corynebacterium* species from human skin (MD Collins et al. [5]). Although this species presents meso-DAP, arabinose and galactose in the cell wall, it does not contain mycolic acids that are found in the structure of all other members of this genus. Despite this discrepancy, 16S ribosomal DNA sequence analysis placed the new species in the genus *Corynebacterium*. The organism was named *C. amycolatum* (i.e. without mycolic acids). Various studies have shown that *C. amycolatum*, was previously misidentified by the clinical laboratories as *C. striatum*, *C. xerosis*, *C. minutissimum* or CDC group I-1 (C. Barreau et al. [6], G. Funke et al. [7], AS. Zinkernagel et al. [8]). *C. amycolatum* was afterwards isolated from severe human infections such as bacteremia and endocarditis (A. Dalal et al. [9], S. Yoon et al. [10], J. Belmares et al. [11]). *C. striatum,* a species found in cattle, is part of normal microbiota of the nose and is also a transient colonizer of the human skin. Since 1993, there have been reported cases of infection with *C. striatum* in immunocompromised individuals leading to the conclusion that this species can be considered an emerging pathogen for this group of patients (P. Cowling et al. [12]).

C. urealyticum was known as CDC group D2, and in 1992, the name of *C. urealyticum* (JM. Aguado et al. [13], D. Pitcher et al. [14]) was proposed and adopted. This species has been associated with acute or chronic infections of the urinary tract, urolithiasis, renal calculi and ureteric stenosis (B. Dominguez-Gill et al.[15], T. Nebreda-Mayoral et al. [16], JM. Aguado et al. [17], F. Soriano et al. [18], F. Soriano et al. [19]). Infection with *C. urealyticum* is associated with alkaline encrusted cystitis. Although considered a disease of the urinary tract, *C. urealyticum* has been isolated from blood culture bacteremia, endocarditis, pericarditis, osteomyelitis, wounds and soft tissue infections (M. Chomarat et al. [20], J. Berenguer et al. [21], I. Fernández-Natal et al. [22], M. Ojeda-Vargas et al. [23], J. Saavedra et al. [24], CA. Wood et al. [25]). *C. afermentans* is a species described by Riegel in 1993. Previously, strains of this species were classified in *CDC group ANF-1* (afermentative corynebacteria). *C. afermentans* contains two subspecies: *C. afermentans subsp. afermentans* and *C. afermentans subsp. lipophilum* **(**P. Riegel et al. [26]). In 1995, the first case of endocarditis due to *Corynebacterium afermentans subsp. lipophilum* (DL. Sewell et al. [27]) was described and also in 1995 there was a report of a case of infection caused by *C. afermentans* manifested by multiple abscess localized in brain and liver in a man of 39 years without medical history. Liver nodules have penetrated the diaphragm causing pleural effusion and periostitis rib (S.Roelf et al. [28]). In 2004 a case of pleuropulmonary necrotizing infection in a patient infected with HIV was described as determined by *C. afermentans subsp. lipophilum* (R. Minkin et al. [29]). *Corynebacterium pseudodiphtheriticum* is part of the normal oropharyngeal flora of the human respiratory tract. Infections associated with this species include exudative pharyngitis, bronchitis, bronchiolitis, necrotizing tracheitis, tracheobronchitis, pneumonia and lung abscess (A. Kamruddin et al. [30], RH. Andavolu et al. [31], E. Chiner et al. [32], HG. Colt et al. [33], JD. Freeman et al. [34]). It was also involved in the etiology of native valve endocarditis in patients with preexisting valvular lesions (A. Morris et al. [35], ME.Wilson et al. [36]). In Romania, the epidemiological data regarding the involvement of *Corynebacterium* strains in infectious pathology is scarce. To the best of our knowledge, the only existing studies concern three isolates of *Corynebacterium urealyticum* recovered from child urine in 1997 (G. Coman et al. [37]), a strain of *Corynebacterium striatum* isolated from a lung infection (D. Diculencu et al. [38]) and *Corynebacterium striatum/amycolatum* strains isolated in ventilator-associated pneumonia at the Cardiovascular Surgery Clinic of Iaşi (RI. Serban et al. [39]).

The aim of our study is to isolate, identify and characterize *Corynebacterium* spp. from Romanian patients using phenotypic and molecular methods.

2. Material and methods

Bacterial Strains

Twenty *Corynebacterium* spp. isolates from blood culture (n=6), peritoneal fluid (n=2), urine (n=3), wounds (n=5), drainage catheter (n=1), perirectal abcess (n=1), osteomyelitis (n=1) and conjunctival secretions (n=1) were analysed. The origin of isolates is presented in Table 1. The isolation of *Corynebacterium* strains was carried out on Columbia Bood Agar supplemented with 7% sheep blood and incubated aerobically for 24-48 h at 37°C.

Characteristic colonies were analyzed by standard microbiological methods: Gram stain, catalase and oxidase tests.

Biochemical characterization

All isolates were biochemically characterized using the commercial API Coryne kit according to the manufacturer's instructions, Maldi-toff method and final identification of species were realized with 16S rRNA gene sequences analysis.

DNA extraction

Bacterial DNA was extracted by thermal lysis method. One loop of overnight culture on Columbia agar supplemented with 7% sheep blood was suspended in 200 µl of sterile distilled water and boiled for 15 min. After boiling, the bacterial suspension was centrifuged and the supernatant was used as a source of template DNA for PCRs.

Amplification of 16S rRNA gene

Each PCR reaction was performed in a final volume of 50 µl reaction mix consisting of: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each of deoxynucleoside triphosphate, 1 U *Taq* DNA polymerase (Invitrogen), 0.4 µM of each primer:

16S Ad: 5′- AGAGTTTGATCMTGGCTCAG -3′(M= C or A),

16S Rj: 5′- AGAGTTTGATCMTGGCTCAG -3′) (M. Oprea et al.[40], WG. Weisburg et al.[41]) and 5 µl of bacterial lysate. After an initial denaturation at 94°C for 4 min, 35 amplification cycles were completed in the thermal cycler. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 49°C, and 1 min of extension at 72°C. A final extension for 7 min was carried out at 72 °C. PCR products (1400 bp length) were purified using WIZARD SV Gel and PCR Clean Up System kit.

Sequencing of 16S rRNA gene amplicons

The amplicons were sequenced on both strands using the primers 16S

Ad 5'–AGAGTTTGATCMTGGCTCAG-3' (M= C or A) and

rE 5' - GGACTACCAGGGTATCTAAT – 3' (M. Oprea et al. [40], WG. Weisburg et al. [41]), and Big Dye terminator cycle sequencing kit as recommended by manufacturer on an Applied BioSystems model 3100 *Avant* automated DNA sequencing system.

The consensus sequences were searched against the GenBank database by using the BLAST tool and were submitted to the Ribosomal Database Project for similarity ranking.

3. Results

All isolates were primarily identified using API Coryne systems. "Very good identification" profiles corresponding to the following species codes: *C. urealyticum* (n=3), *C. afermentans* (n=2), *C. pseudodiphtheriticum* (n=1) and *C. striatum/amycolatum* (n=5) were obtained. For the *C. striatum/amycolatum* strains, it was necessary to apply another method of identification, because API Coryne cannot discriminate between these two species. For the other three strains API Coryne profiles were "inacceptable" or "low discrimination". Species-level identification by phenotypic methods was successful for 6 of 20 isolates and, for genus level

identification, it was successful for all isolates. The results obtained by MALDI-TOF method and the RNA 16 S sequence analysis were similar for eighteen of the twenty strains studied.

Molecular identification using 16S rRNA gene sequences analysis was carried out for the all isolates. Results of identification with API Coryne systems, MALDI-TOF and 16S rRNA sequences analysis are shown in table 1.

Table 1. Results of identification using API Coryne, ARN 16 S gene sequences and MALDI-TOF systems

Strain no.	**Patient description**	**Samples**	**Identification Code API Coryne**	**Identification of API Coryne**	**Identification of MALDI TOF**	**ARN 16 S**
1.	admitted	blood culture I	2100304	*C. jeikeium*	*C. jeikeium*	*C. amycolatum*
2.	admitted	blood culture II	2100004	*C. afermentans*	*C. afermentans*	*C. afermentans*
3.	admitted	blood culture III	2100004	*C. afermentans*	*C. afermentans*	*C. afermentans*
4.	admitted	blood culture IV	1100105	*C. macginleyi*	*C. striatum*	*C. striatum*
5.	admitted	blood culture V	2100304	*C. jeikeium*	*C. jeikeium*	*C. amycolatum*
6.	admitted	blood culture VI	3100125	*C. striatum/amycolatum*	*C. striatum*	*C. striatum*
7.	admitted	peritoneal fluid I	0100324	*C. jeikeium*	*C. amycolatum*	*C. amycolatum*
8.	admitted	peritoneal fluid II	3100324	*C. striatum/amycolatum*	*C. amycolatum*	*C. amycolatum*
9.	admitted	catheter	3100125	*C. striatum/ amycolatum*	*C. striatum*	*C. striatum*
10.	outpatient	osteomyelitis	3000324	*C. accolens* (53%) *C. striatum/ amycolatum* (39.8%)	*C. amycolatum*	*C. amycolatum*
11.	admitted	perirectal abcess	3100324	*C. striatum/amycolatum*	*C. amycolatum*	*C. amycolatum*
12.	outpatient	urine	1100324	*Corynebacterium macginleyi* (46.1%) *Corynebacterium striatum/ amycolatum* (33.2)	*C. amycolatum*	*C. amycolatum*
13.	outpatient	urine	2101004	*Corynebacterium urealyticum*	*C. urealyticum*	*C. urealyticum*
14.	outpatient	urine	2041004	*Corynebacterium urealyticum*	*C. urealyticum*	*C. urealyticum*
15.	outpatient	wound	3100365	*Corynebacterium striatum/ amycolatum* (64.3%) *Corynebacterium* group G (33.9%)	*C. striatum*	*C. striatum*
16.	outpatient	wound	3100105	*C. striatum/ amycolatum*	*C. striatum*	*C. striatum*
17.	admitted	wound	2041004	*C. urealyticum*	*C. urealyticum*	*C. urealyticum*
18.	admitted	wound	1100105	*C. macginleyi*	*C. striatum*	*C. striatum*
19.	admitted	wound	1100105	*C. macginleyi*	*C. striatum*	*C. striatum*
20.	outpatient	conjunctival secretion	3101004	*Corynebacterium pseudodiphtheriticum*	*Corynebacterium pseudodiphtheriticum*	*Corynebacterium pseudodiphtheriticum*

4. Discussion

In recent years numerous works have shown the involvement of several species belonging to the genus *Corynebacterium*, other than *C. diphtheriae* classic pathogen of the genus, in superficial and invasive infections (A. Dalal et al. [9], S. Yoon et al. [10], J. Belmares et al. [11]), P. Cowling et al. [12], B. Dominguez-Gill et al.[15], T. Nebreda-Mayoral et al. [16], JM. Aguado et al. [17], F. Soriano et al. [18], F. Soriano et al. [19], M. Chomarat et al. [20], J. Berenguer et al. [21], I. Fernández-Natal et al. [22], M. Ojeda-Vargas et al. [23], J. Saavedra et al. [24], CA. Wood et al. [25], DL. Sewell et al. [27], S.Roelf et al. [28], (R. Minkin et al. [29], A. Kamruddin et al. [30], RH. Andavolu et al. [31], E. Chiner et al. [32], HG. Colt et al. [33], JD. Freeman et al. [34], A. Morris et al. [35], ME.Wilson et al. [36]). Because the normal habitat for this species is the human skin and mucous membranes, they are therefore sometimes isolated as contaminants in clinical samples (F.N.R. Renaud et al. [4]). In a review Funke et al. affirm: "Identification of coryneform bacteria to the species level often causes problems but should be performed whenever they grow in pure culture from clinical specimens and/or when they represent the predominant organisms in normally sterile samples" (G. Funke et al. [2]). Our study confirms the latter observations and, at the same time, demonstrates that these etiological agents can be also found in patients with different pathologies. The strains isolated from urine samples grew in pure culture, in significant quantity (> 10^5 CFU / ml), the strains isolated from other samples were the predominant organisms in culture and we could isolate the same strains in two or three successive samples, *e.g.* infection isolates from the drainage catheter post-surgical intervention *C. striatum* was predominantly isolated in three successive determinations. Our exhaustive sample evaluation in the last two years, showed that even very few, there still are infections caused by strains belonging to the genus *Corynebacterium*, in Romania. Most previously published studies reveal that the efficacy of biochemical identification systems is limited, and there is a need to perform additional tests for an accurate species level identification. In an evaluation of the API Coryne system, 90.5% of 407 strains of coryneform bacteria were correctly identified (G. Funke et al. [42]). Additional tests were required to definitively identify 55.1% of these isolates; however, 5.6% of strains were not identified, and 3.8% of strains were misidentified (G. Funke et al.[42]). Due to the small number of isolated strains, we cannot have statistical inference from our study. The identification of coryneform bacteria from clinical isolates is difficult. For the accurate species identification, molecular techniques are needed, namely 16S ribosomal DNA gene analysis. While the availability of commercial biochemical panels and nucleic acid amplification techniques has enabled diagnostic laboratories to identify most bacterial pathogens with great accuracy and expediency, their relative efficacies in classifying more unusual opportunistic pathogens are less well described. Analysis of 16S rRNA gene sequences offers a reliable tool for organism identification (YW. Tang et al. [43]) and routine use of this method should increase our knowledge regarding the clinical spectrum of *Corynebacterium* infections in human subjects. In our country, infections caused by corynebacteria often remain undiagnosed because these species especially *C. urealyticum,* grow more slowly on ordinary culture media, or may be incorrectly classified.

5. Conclusion

Our study illustrates the difficulties in identification of *Corynebacterium* species in the routine laboratory activity and for final identification of non-diphtheriae *Corynebacterium* spp. Starting from clinical samples, a combination of phenotypic and molecular – biology -

based identification techniques is necessary. Our results may be useful for clinicians and hospital laboratories on the involvement of these strains in infectious pathology, focusing on isolation and accurate classification within *Corynebacterium* genus and for establishing the best therapeutic treatment. In order to better evaluate the real incidence of non-diphtheriae *Corynebacterium* spp. in our country, a very good collaboration between clinicians, medical laboratory staff and public health specialists is needed.

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Conflict of interest

The authors declare that they have no conflict of interest.

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