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Identification of Clinical *Corynebacterium striatum* Strains by PCR-Restriction Analysis Using the RNA Polymerase β-subunit gene *(rpob)*

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Abstract

Corynebacterium striatum is frequently encountered in the routine clinical microbiology laboratory. It is widely disseminated in the environment and constitutes part of the normal micro-biota of the skin and mucous membrane. Identification of this species by biochemical methods remains difficult and several misidentifications have been reported previously. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for the identification of this microorganism was designed based on the hypervariable region of the polymorphic RNA polymerase β-subunit gene (*rpoB*). All available *Corynebacterium rpoB* sequences were analyzed by computer-assisted restriction analysis. The *rpoB* PCR-RFLP pattern predicted by using endonucleases *MseI* and *NlaIV* clearly differentiated *C. striatum* from all other *Corynebacterium* species. This method was successfully applied for the reliable identification of 67 *C. striatum* clinical isolates and can be used for the timely detection of infected patients or for epidemiological studies.

Keywords: *Corynebacterium striatum*; Identification; *rpoB*; RFLP

Introduction

The genus *Corynebacterium* is composed of Gram positive bacteria, facultative anaerobe, that are widely distributed [1]. The identification of *Corynebacterium* species is difficult because it always needs particular techniques or a big number of biochemical tests that are not available in API system [2]. Several molecular methods have been used to identify *Corynebacterium* species including DNA-DNA hybridization [3], sequence analyses of *16S rRNA* and *rpoB* genes [4] and *rpoB* gene RFLP [1]. However, the *16S rRNA* genes sequence analysis, which is the most used to identify bacteria or to determine their phylogenetic relationships, has limits in the identification of *Corynebacterium* species because of his low intragenus polymorphism. The *rpoB* gene is polymorphic enough to be used for the accurate identification of *Corynebacterium* species [5]. Pavan et al. [1] demonstrated that *rpoB* RFLP analysis can be used for the reliable identification of *C. pseudotuberculosis* strains isolated from sheep. In our study, we investigated the application of PCR-RFLP analysis of the hypervariable sequence of *rpoB* gene for the speciation of *Corynebacterium striatum* strains (Figure 1).

Material and Methods

Bacterial strains

Eighty five strains identified as *C. striatum/amycolatum* by the routine assays and Api Coryne V.2 strips were studied. Four others clinically relevant *Corynebacterium spp*: *C. macginleyi, C. diphtheriae, C. coylae* and *C. jeikeuim* were included in this study. The strains were collected from multiple clinical sources, including blood, tissue, urine, wound, respiratory specimens and others sources, during a period of five years (2007-2013) in the Universitary Hospital F. Hached, Tunisia. *C. striatum* ATCC6940 was used as control.

Figure 1: Schematic representation of the *rpoB* gene showing the restriction sites of the endonucleases for *C. striatum*.

Choice of restriction enzymes

Initially all the *rpoB* hypervariable sequences publicly available in GenBank database belonging to 62 *Corynebacterium* species were aligned [1]. Enzyme restriction patterns for the *rpoB* amplified region of each *Corynebacterium* species were generated using REBASE program [6]. The predicted *MseI* and *NlaIV* restriction fragments of the *rpoB* amplicon in *Corynebacterium* species using REBASE program are listed in Table 1. The majority of *Corynebacterium* species did not contain a restriction site for these endonucleases.

rpoB PCR-RFLP analysis

All strains were cultured overnight, on 5% horse blood agar in 5% CO_2 at 37°C. The DNA from each strain was extracted by QIAamp DNA MiniKit (Qiagen GmbH, Germany), following the manufacturer's instructions. PCR was carried out in a final volume of 50 µl as described previously using oligonucleotides C2700F and C3130R [1,5]. Amplified products were separated in agarose gel 2% and were visualized by ethidium bromide staining. Following the PCR, 12 µl of amplified products were digested using endonucleases *MseI* and *NlaIV* in two separate reactions according to manufacturer's

guidelines. RFLP products were analyzed using 2% agarose gel, at 100V for 1 hour. A 100-pb molecular weight marker was used as a molecular size standard (Figure 2).

Species and *rpoB* Genbank acession number	**Predicted sizes of fragments (bp) after amplicon digestion with:**	
	MseI	*NlaIV*
C. striatum AY492267	323-123	138-308
C. simulans AY492264	nr	138-308
C. ulcerans AY492271	nr	nr
C. amycolatum AY492241	nr	nr
C. minutissimum AY492235	nr	369-77
C. maginleyi AY492276	98-348	278-91-77
C. pseudotuberculosis AY492239	351-95	nr
C. glucuronolyticum AY492256, C. seminale AY492263	311-123	nr
C. imitans AY492259	nr	nr
No restriction sites for these enzymes were found in the *rpoB* sequences of the following *Corynebacterium* species: *C. accolens* AY492242, *C. ammoniagenes* AY492243, *C. argentoratense* AY492249, *C. aurimucosum* AY492282, *C. auris* AY492234, *C. auriscanis* AY492244, *C. bovis* AY492236, *C. camporealensis* AY492246, *C. capitovis* AY492247, *C. casei* EU616817, *C. confusum* AY492248, *C. coyleae* AY492250, *C. diphtheriae* AY492230, *C. durum* AY492252, *C. efficiens* AP005215, *C. falsenii* AY492253, *C. felinum* AY492254, *C. flavescens* AY492255, *C. freneyi* AY492237, *C. genitalium* EU616818, *C. jeikeium* AY492231, *C. lipophiloflavum* AY492260, *C. mastitidis* AY492281, *C. matruchotii* AY492238, *C. mucifaciens* AY492261, *C. mycetoides* AY492262, *C. phocae* AY492277, *C. propinquum* AY492279, *C. pseudodiphtheriticum* AY492232, *C. pseudogenitalium* AY581868, *C. pyruviciproducens* FJ899747, *C. riegelii* AY492278, *C. singulare* AY492280, *C. spheniscorum* AY492283, *C. sundsvallense* AY492268, *C. terpenotabidum* AY492269, C. testudinoris AY492284, *C. thomssenii* AY492270, *C. tuberculostearicum* AY581869, *C. urealyticum* AY492275, *C. ureicelerivorans* FJ392022/ FJ392020/ FJ392018/ FJ392029/FJ392021, *C. variabile* AY492272, *C. xerosis* AY492233.		

Table 1: Predicted *MseI* and *NlaIV* restriction fragments of the *rpoB* amplicon in different *Corynebacterium* species using the REBASE program.

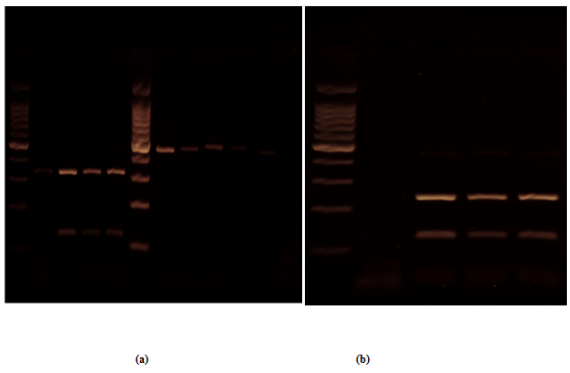

Figure 2: Identification of *Corynebacterium* striatum strains by *rpoB*-PCR-RFLP. **(a)** Lane 1 and 6: 100 bp ladders. Lane 2-5: *rpoB* gene *MseI* restriction from *C. striatum*. Lane 7-11: *rpoB* gene *MseI* restriction from *C. amycolatum*, *C. singulare*, *C. aurimucosum*, *C. simulans* and *C. imitans* respectively. Lane 12: Negative control (5 µl of water instead of DNA). **(b)** Lane 1: 100 bp ladder. Lane 2: Negative control. Lane 3-4: *rpoB* gene *NlaIV* restriction from *C. imitans* (n=1) and *C. striatum* (n=2).

MALDI-TOF-MS analyses

To confirm the identification, all strains were analyzed by MALDI TOF MS (Bruker Daltonics, GmbH) as previously described [7]. Briefly, a portion of a colony was smeared onto a 96-well target plate, and after drying, it was covered using 1 µl of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution. When it was dry, the target plate was loaded into the machine, which was equipped with a 337-nm nitrogen laser. The spectra were analyzed using the Biotyper 2.0 software (Bruker, Karlsruhe, Germany). The identification criteria were chosen according to the cutoffs proposed by the manufacturers. Identifications with scores above 2 and between 1.7 and 2 were considered to be reliable at the species and genus levels, respectively. Identification scores below 1.7 were considered unacceptable. When the MALDI-TOF-MS identification was inconclusive, 16S *rRNA* gene sequencing was performed.

Results and Discussion

As a result of their being normal human microbiota, *Corynebacterium* species are commonly considered as contaminants. Because of this and of challenges in identification, they have not received a great deal of attention [8]. Identification of putative pathogenic *Corynebacterium* is crucial. So far, this has been done biochemically, with Api Coryne strips which takes at least 16 hours after isolation of suspicious colonies from screening plates (typically small grayish colonies, mostly translucent, positive catalase reaction and Gram positive coryneform rods in the form of Chinese letters), and may often yielded unreliable or ambiguous results [7,8]. In this

study, we distinguish 2 different colonies' morphology. Seventy one isolates produced on Columbia agar base with 5% horse blood non-hemolytic, creamy white to yellowish with an entire edge colonies. However, for 15 isolates, colonies were flat, dry, whitish-gray and matte. Using biochemical tests, these strains were identified as *C. striatum/amycolatum*. In Api Coryne database *C. amycolatum, C. minutissimum*, and *C. striatum* gave the same code: (2-3)100(1-3)(0-2)(4-5). Their differential identification by biochemical tests remains difficult, and several misidentifications have been previously reported [9-11]. Although they are genetically different, these species share many phenotypic characteristics and we need supplementary tests to differentiate them [12]. The RFLP analysis of the amplified sequence of *Corynebacterium* strains identified as *C. striatum/amycolatum* by Api Coryne strips indicated that 67 strains presented exactly the same *MseI* and *NlaVI* restriction fragments corresponding to predicted patterns for *C. striatum*. By MALDI-TOF-MS identification, these strains were assigned to *C. striatum* with scores >2.000. However, 18 strains identified as *C. striatum/amycolatum* by Api Coryne did not contain restriction sites for *MseI* and *NlaIV*. These strains were identified as *C. amycolatum* (n=14), *C. aurimucosum* (n=2), *C. imitans* (n=1) and *C. singulare* (n=1). The assay was also successfully applied to differentiate *C. striatum* from other clinically relevant *Corynebacterium* spp. including *C. macginleyi*, *C. diphtheriae*, *C. jeikeuim* and *C. coylae/afermentans* (Table 2). Furthermore, the technique proposed has the potential to differentiate C. striatum from the other biochemically and genetically related *Corynebacterium* spp: *C. amycolatum, C. minutissimum, C. simulans, C. ulcerans* and *C. aurimucosum*, none of which have the same restriction sites for *NlaIV* or *MseI* in the *rpoB* region analyzed. It must be taken into account that several base changes would be required in order to change the restriction sites so that a strain from other *Corynebacterium* species acquired the pattern of *C. striatum*.

Strains identified by n			**Final identification**
Api Coryne Strips (number of strains n)	PCR-RFLP-*rpoB* gene profile		
	MseI	*NlaIV*	
C. striatum/amycolatum (n= 67)	323-123	138-308	67 *C. striatum*
C. striatum/amycolatum (n=18)	NR	NR	14 *C. amycolatum* 2 *C. aurimucosum* 1 *C. singulare* 1 *C. imitans*
C. macginleyi (n=1)	98-348	278-91-77	1 *C. macginleyi*
C. diphtheriae (n=1)	NR	NR	1 *C. diphtheriae*
C. jeikeuim (n=1)	NR	NR	1 *C. jeikeuim*
C. coylae/afermentans (n=1)	NR	NR	1 *C. coylae*

Table 2: Comparison of the results obtained using different methods of identification.

The present study proposes a molecular method involving the PCR-mediated amplification of an internal *rpoB* region followed by RFLP-analysis. This method has been designed for the identification of *C. striatum*, distinguishing it from other *Corynebacterium* species, including all members of this genus of importance in clinical medicine, and from genetically related pathogens such as *C. simulans* and *C. ulcerans*. The PCR-RFLP technique described in this work has been experimentally tested to differentiate *C. striatum* from *C. amycolatum* and *C. minutissimum* that produces similar Api Coryne codes. This assay provides a rapider diagnostic tool than biochemical assays or 16S RNA sequencing, for the identification of clinical *C. striatum* strains and for the discrimination between this species and other related pathogenic bacteria.

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