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Note (REVISED)

Efficient differentiation of *Corynebacterium striatum*, *Corynebacterium amycolatum* and *Corynebacterium xerosis* clinical isolates by multiplex PCR using novel species-specific primers

Carolina S. Santos [a], Juliana N. Ramos [b], Veronica V. Vieira [c], Carina S. Pinheiro [a], Roberto Meyer [a], Neuza M. Alcantara-Neves [a], Rommel T. Ramos [d], Artur Silva [d], Raphael Hirata Jr. [e], Liza Felicori [f], Carlos Ruiz de Alegría Puig [g], Jesús Navas [g], Vasco Azevedo [f], Ana L. Mattos-Guaraldi [e], Luis G. C. Pacheco [a,*]

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Keywords: Emerging pathogens; *Corynebacterium* spp.; Bacterial identification; Multiplex PCR; Vitek-MS.

Abstract

A multiplex-PCR (mPCR) assay was designed with species-specific primers which generate amplicons of 226 bp, 434 bp and 106 bp for differentiating the species *C. striatum*, *C. amycolatum*, and *C. xerosis*, respectively. mPCR results were 100% in agreement with identifications achieved by *16S rRNA* and *rpoB* gene sequencing and by VITEK-MS.

In recent years there has been a considerable increase in the numbers of reports of nondiphtherial corynebacteria as the causative agents of opportunistic and nosocomial infections in humans (Díez-Aguilar et al., 2013; Chandran et al., 2016; Wolcott et al., 2016). Although these microorganisms are constituents of the normal skin and mucous membranes human microbiota, their clinical relevance as emerging human pathogenic species has been demonstrated by separate studies (Leal et al., 2016; Mushtaq et al., 2016). Noteworthy, the rapid emergence of multidrug-resistant strains has been recently reported for the species *Corynebacterium striatum* (Werth et al., 2016; Hahn et al., 2016).

An important cause for under-diagnosis of infections caused by emerging *Corynebacterium* spp. is the difficulty to identify isolates to the species level. Accurate identification is essential for correct disease diagnosis and treatment of infection. In clinical microbiology laboratories, these microorganisms are routinely identified with the API Coryne system (bioMérieux) and complementary phenotypic tests (Bernard, 2012). However, this method does not always provide reliable identification at the species level. In fact, some *Corynebacterium* species are considered as "difficult-to-identify" by traditional biochemical tests. The current version of the API Coryne system does not differentiate between the species *C. striatum* and *Corynebacterium amycolatum*. Besides, *Corynebacterium xerosis*, another species generating ambiguous identifications (Bernard, 2012), is not present in the APIweb™ database. Additional tests should be employed for efficient identification of these species, including susceptibility to the vibriostatic agent O/129 for differentiation between *C. striatum* and *C. amycolatum* (Alibi et al., 2015a), and molecular identification by *16S rRNA* and *rpoB* gene sequencing (Bernard, 2012), which is slow and expensive. Identification by MALDI-TOF mass spectrometry has also proven very effective for correct species assignment of nondiphtherial corynebacteria (Alibi et al., 2015a; Leal et al., 2016; Mushtaq et al., 2016; Alibi et al., 2017). However, while this technology is still not fully accessible to clinical microbiology laboratories in developing countries, some "difficult-to-identify"

Corynebacterium species might still require additional molecular tests for accurate identification; this includes differentiation between the species *C. amycolatum* and *C. xerosis* (Leal et al., 2016; Alibi et al., 2017).

Herein, we have developed a multiplex PCR method based on species-distinctive genes for accurate identification at the species level of *C. striatum*, *C. amycolatum* and *C. xerosis*. Species-specific target genes were detected by whole-genome comparisons between recently sequenced isolates (Pacheco et al., 2015; Mattos-Guaraldi et al., 2015). In brief, comparative genome analyses were performed through the software platforms EDGAR 2.0 (http://edgar.computational.bio) and SEED Viewer (http://pubseed.theseed.org); hypothetical genes were filtered out and the remaining annotated unique genes for each species were further interrogated using NCBI's BlastN searches for definition of PCR targets. Primer design was done with the Primer-Blast tool and specificity checking was achieved through *in silico* PCR analysis against a custom database containing 3,417 genomic sequences (comprising contigs, scaffolds and chromosomes) pertaining to 50 strains of 15 different emerging pathogenic nondiphtherial *Corynebacterium* species.

Table 1 shows the chosen target genes and species-specific primer pairs that could provide efficient differentiation between the species *C. striatum*, *C. amycolatum* and *C. xerosis*. Results of *in silico* PCR analysis showed 100% sensitivity of the newly designed primers; specificity varied between 94.0% - 100% and this was, in part, due to incorrect species assignment of some genomic sequences retrieved from public databases, particularly for strains of *C. jeikeium* and *C. urealyticum*. Additional primer pairs were also designed for the species *Corynebacterium minutissimum* but showed poor sensitivities and specificities (Supplementary Table S1); this might also be partially attributed to the existence of incorrectly identified genomic sequences of *C. minutissimum* and of the difficult-to-distinguish species *Corynebacterium aurimucosum* and *Corynebacterium singulare*.

The chosen species-specific primers were then used in PCR reactions with a panel of 29 bacterial clinical isolates of the *C.striatum*/ *amycolatum*/ *xerosis* group and related species (cross-reactivity controls), mostly presenting variable biochemical profiles. The 29 isolates had been previously identified using API Coryne and Vitek 2, *16S rRNA* and *rpoB* gene sequencing, and VITEK-MS (Table 2). Fifteen-microliters PCR reactions contained: 0.025 U/µL *Pfu* DNA polymerase (Promega); 1X *Pfu* buffer with $MgSO_4$; 0.2 mM dNTP mix; 0.75 µM each primer; at least 15 ng of template DNA. Cycling was in a ProFlex 3 instrument (Thermo), as follows: 30 cycles of denaturation at 94ºC for 30 s, primer annealing at 63°C for 40 s, and extension at 72ºC for 50s. Reactions performed well both in conventional singleplex and multiplex formats.

Despite the discrepancies observed when compared to biochemical identification methods, the results obtained with the novel mPCR assay were 100% in agreement (Kappa = 1.0) with identifications achieved by *16S rRNA* and *rpoB* gene sequencing and by VITEK-MS (correctly assigned species = 21/21 isolates; incorrectly assigned species = 0/29 isolates, including *C. minutissimum*) (Table 2). All isolates categorized by VITEK-MS as *C.amycolatum*/*xerosis* (n = 4) were identified as *C. amycolatum* by mPCR. Previous attempts to identify these corynebacterial species using PCR-based approaches either required complementary analyses, such as digestion with restriction endonucleases (Sierra et al., 2005; Alibi et al., 2015b), or needed more complex interpretation of results (Letek et al., 2006; Cazanave et al., 2012). This illustrates the usefulness of the newly developed mPCR with species-specific primers as a rapid complementary approach for resolving ambiguous or incorrect identifications of species of the *C.striatum*/*amycolatum*/*xerosis* group. However, analysis with an expanded panel of isolates will be necessary to accurately determine the diagnostic value of this assay. Besides, the availability of a mPCR assay that could also include the species *C. minutissimum* would be highly desirable. This was hampered in this study due to the inability to identify unique genes that could specifically detect this species without cross-reacting with related bacteria. This goal will potentially be achieved as novel genomic

sequences for the species *C. minutissimum* and related species, such as *C. aurimucosum* and *C. singulare*, become available.

Conflict of interests

No potential conflicts of interest were disclosed.

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Table 1. Species-specific primer pairs used in multiplex-PCR and results of *in silico* PCR analysis

Species	Gene description (GenBank ID)[a]	Amplicon size (bp)	Species-specific primers (5'- 3') [a]	*In silico* PCR results [b]
C. striatum	Putative ferrous iron transporter (EEI79352.1)	226	Cst_1-F: CTTCGAAGAACATGAAGGCA Cst_1-R: CCGTAGTACATCGCTACGGC	**10 / 10 *C. striatum*** (100% sensitivity) **0 / 40 other emerging *Corynebacterium* spp.** (100% specificity)
C. amycolatum	Alpha/beta hydrolase (WP_005511598)	434	Camy_2-F: ATGACCAAGACGGATGCTGG Camy_2-R: TTGGTGGGAACGAAACCACA	**3 / 3 *C. amycolatum*** (100% sensitivity) **3 / 47 other emerging *Corynebacterium* spp.** (94.0 % specificity) [c]
C. xerosis	Permease of the drug/metabolite transporter superfamily (WP_046650372)	106	Cxer-F: CGTGGACGAGGCTGGAATC Cxer-R: ACCAGTTTCGGGGTGAGGA	**2 / 2 *C. xerosis*** (100% sensitivity) **2 / 48 other emerging *Corynebacterium* spp.** (96.0 % specificity) [d]

[a] Primers designed after comparative whole-genome analysis of the following genomic sequences: *C. striatum* 1961 BR-RJ/09; *C. amycolatum* SK46; *C. xerosis* ATCC 373T; *C. minutissimum* 1941. Interspecies comparison of gene content was achieved with EDGAR software (http://edgar.computational.bio). Species-specific gene selection (four strains comparison) and filtering out of hypothetical genes and mobile genetic elements was done with SEED Viewer comparative tool (http://pubseed.theseed.org) and by manual curation. Specificity checking was performed with megaBLAST against a database containing 28,937 sequences of *Corynebacterium* spp. (including contigs, scaffolds and chromosomes).

[b] *In silico* PCR was performed against a custom database containing 3,417 genomic sequences (comprising contigs, scaffolds, and chromosomes) from 50 strains representing 15 emerging *Corynebacterium* species: *C. striatum* (n = 10); *C. amycolatum* (n = 3); *C. xerosis* (n = 2); *C. minutissimum* (n = 3); *C. aurimucosum* (n = 6); *C. propinquum* (n = 4); *C. pseudodiphtheriticum* (n = 2); *C. argentoratense* (n = 3); *C. singulare* (n = 1); *C. simulans* (n = 3); *C. afermentans* (n = 2); *C. coyleae* (n = 1); *C .freneyi* (n = 1); *C. jeikeium* (n = 6); *C. urealyticum* (n = 3). Parameters: (i) mismatch threshold: up to 6 mismatches allowed; (ii) targets with mismatches at extreme 3'-end were discarded; (iii) max. amplicon size of 500 bp.

[c] The three strains that showed potential amplification products with the *C. amycolatum* primers were: *C. urealyticum* strain 1055_CURE, and *C. jeikeium* strains 212 and 805. The genomic sequences of these strains show high average nucleotide identities (ANIb) and TETRA values, when compared with a *C. amycolatum* reference strain (data not shown). This indicates they represent potentially misidentified sequence entries on GenBank, and might therefore belong to the species *C. amycolatum.*

[d] The two unintended targets presented 5 mismatches, being one in the second base at the extreme 3'-end.

Table 2. Results of mPCR with species-specific primers in comparison to other identification methods of 29 bacterial isolates

Bacterial isolates [a]	***16S rRNA*** **+** ***rpoB*** [b] **or VITEK-MS** [c]	**API Coryne**	**Vitek 2**	**mPCR** [d] **(positives)**
C. striatum (n = 8) 2103, 2308, 2324, 2376, 2390, 2401, 2432, 2454	*C. striatum* (8)	*C. striatum/ amycolatum* (3) *C. argentoratense* (1) *C. afermentans/ coyleae* (1) *C. macginley* (1) NA (2)	*C. striatum* (5) *C. minutissimum* (2) NA (1)	***C. striatum*** **(8)** *C. amycolatum* (0) *C. xerosis* (0)
C. amycolatum (n = 12) 2354, 2407, 2459, 2125A, 2255, 2298, 2305, 2404, 13-1219, SA60, SA90, SA127	*C. amycolatum* (8) *C.amycolatum /xerosis* (4)	*C. striatum/ amycolatum* (7) *Corynebacterium* group G (2) *C. jeikeium* (2) NA (1)	*C. amycolatum* (6) *C. jeikeium* (2) NA (4)	*C. striatum* (0) ***C. amycolatum*** **(12)** *C. xerosis* (0)
C. xerosis (n = 1) ATCC373^T	*C. xerosis* (1)	NA	NA	*C. striatum* (0) *C. amycolatum* (0) ***C. xerosis*** **(1)**
C. minutissimum (n = 1) 2422 *C. aurimucosum* (n = 4) 15-4203, 15-4290, 15-6769, 16-3925 *C. jeikeium* (n = 1) 14-0806 *Corynebacterium sp.* (n =1) 15-1068 *Brevibacterium luteolum* (n =1) 14-3553	*C. minutissimum* (1) *C. aurimucosum* (4) *C. jeikeium* (1) *Corynebacterium sp.* (1) *Brevibacterium luteolum* (1)	*C. striatum/ amycolatum* (2) *C. aurimucosum* (4) *Corynebacterium sp.* (2)	NA (8)	*C. striatum* (0) *C. amycolatum* (0) *C. xerosis* (0)

[a] Bacterial isolates were obtained from: The Laboratory of Diphtheria and Corynebacteria of Clinical Importance (LDCIC) and the Laboratory of Bacteriology (LABAC), both from the Rio de Janeiro State University (UERJ), Brazil; Hospital Universitario Marqués de Valdecilla, Santander, Spain; and Hospital Farhat Hached, Sousse, Tunisia. *C. xerosis* ATCC373^T is a type strain for the species.

[b] *16S rRNA* sequences were *ca*. 1,500 bp and showed higher than 98.0% identity to reference; *rpoB* gene sequences were *ca.* 400 bp and showed higher than 97.0% identity to reference.

[c] MALDI-TOF MS-based identifications were performed with VITEK-MS (bioMérieux), as described by Alibi et al (2017). Isolates identified by VITEK-MS are underlined.

[d] PCR reactions were performed with the species-specific primer pairs (Table 1) both in conventional singleplex and multiplex formats, and rendered similar results.

Highlights

Emerging pathogenic *Corynebacterium* species

Multiplex PCR method based on species-distinctive genes

Accurate identification of *C.striatum*, *C.amycolatum* and *C.xerosis*