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A PCR for *dtxR* gene: Application to diagnosis of non-toxigenic and toxigenic *Corynebacterium diphtheriae*

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ABSTRACT

The significant rise in the percentage of adults susceptible to diphtheria and the emergence of non-toxigenic *Corynebacterium diphtheriae* strains as the causative agent of endocarditis and other systemic infections emphasize the need for alternative laboratory diagnostic procedures. In this study, for the first time, the value of a species-specific PCR assay that targets the *dtxR* gene is documented as a procedure for differentiating *C. diphtheriae* from *Corynebacterium*-like colonies. The results of the PCR–dtxR were all positive for 91 *C. diphtheriae* (54 non-toxigenic and 37 toxigenic) strains. PCR–dtxR completely correlated with the standard biochemical and commercial identification for all *C. diphtheriae* strains tested. Conversely, the PCR–dtxR results were negative in 100% of the 111 non-diphtherial Gram-positive rod strains obtained during identification procedures in a hospital laboratory. Thus, the PCR–dtxR assay emerged as viable, cost-effective screening method for *C. diphtheriae* laboratory identification.

1. Introduction

The worldwide persistence of *Corynebacterium diphtheriae* exhibiting genotypic and phenotypic variability requires the application of innovative diagnostic measures. *C. diphtheriae* infection is not easily diagnosed on clinical grounds alone, particularly among individuals vaccinated against diphtheria, especially in cases of diphtheria without pseudomembrane formation [1]. In addition to the increased frequency of cases arising from non-toxigenic strains, potential errors are especially significant in view of the several clinical forms the disease has presented [2,3]. It is, therefore, important to continually upgrade pathogen-identification methodologies [4].

Housekeeping genes encoding proteins that carry out essential cellular functions make excellent targets for species-specific probes [5]. Reported data have confirmed that all *C. diphtheriae* strains studied harbor the *dtxR* gene [3,6]. The protein product of this gene is known to function as a global regulator of metabolism in both toxigenic and non-toxigenic *C. diphtheriae* strains, and includes the regulation of expression of *tox* gene [3,6,7]. DtxR-like transcriptional regulators have been identified by sequence homology in other *Corynebacterium* species [8] and in many bacterial genera, including *Mycobacterium*, *Rhodococcus,* and *Brevibacterium* [9–11].

Until now, there is no data considering a species-specific PCR for *C. diphtheriae* identification without performing additional steps of DNA sequencing or hybridization as previously described [12,13]. The aim of this study was to evaluate the species-specific PCR for the *dtxR* gene as an alternative method for the presumptive identification of *C. diphtheriae* and differentiation from other *Corynebacterium*-like colonies in clinical laboratory procedures.

2. Materials and methods

2.1. Bacterial strains and identification procedures

Ninety-one strains of *C. diphtheriae* (37 sucrose-fermenting and 54 non-sucrose fermenting strains, 80 biotype *mitis*, 5 biotype *gravis*, and 6 biotype *belfanti*, 47 from respiratory tract, 40 from skin, 4 from blood), one of *Corynebacterium ulcerans*, and three of *Corynebacterium pseudotuberculosis* were investigated. All strains were obtained from the culture collection of the Diphtheria Laboratory, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, RJ, Brazil. The present study also included non-diphtherial Gram-positive rods, as follow: *Corynebacterium pseudodiphtheriticum* (19), *Corynebacterium amycolatum* (18), *Corynebacterium afermentans* (16), *Corynebacterium minutissimum* (5), *Corynebacterium glucuronolyticum* (5), *Corynebacterium propinquum* (6), *Corynebacterium striatum* (5), *Corynebacterium jeikeium* (6), *Corynebacterium xerosis* (4), *Corynebacterium accolens* (1), *Corynebacterium urealyticum* (3), *Corynebacterium* sp. (3), *Rhodococcus equi* (6), *Listeria* sp. (3), *Nocardia* sp.

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Table 1
PCR–dtxR, biochemical properties and toxigenicity of *Corynebacterium diphtheriae* and other potentially toxigenic corynebacteria clinical isolates

Species (n. isolates)	API code	Nitrate reduction	Urea hydrolysis	CAMP reaction	PYZ activity	Positive reaction		
						Sucrose fermentation	Toxigenicity[a]	PCR–dtxR[b] %
C. diphtheriae biotype *mitis* (80)	1010124, 1010125, 1010324, 1010325, 1010365	+	−	−	−	32	36	100
C. diphtheriae biotype *belfanti* (6)	0010324, 0010325	−	−	−	−	5	1	100
C. diphtheriae biotype *gravis* (5)	0010326, 1010326	V	−	−	−	0	0	100
Corynebacterium ulcerans (1)	0111326	−	+	REV	−	0	0	0
Corynebacterium pseudotuberculosis (3)	0001324	−	+	REV	−	0	0	0

V, variable; REV, reverse CAMP reaction, PYZ, pyrazinamidase; +, positive reaction; −, negative reaction.
[a] Toxin production by Vero cell citotoxicity assay and *tox* gene detection by PCR-DT.
[b] PCR–dtxR using the four primer sets based on published *dtxR* gene sequences [6].

(7), *Gordona* sp. (1), and *Oerskovia* sp. (3). All non-diphtherial Gram-positive rods strains were isolated from the clinical specimen cultures routinely submitted to the Microbiology Laboratory of the Hospital Universitário Pedro Ernesto (HUPE/UERJ), a 600-bed teaching hospital in the City of Rio de Janeiro.

The following strains were used as controls: toxigenic *C. diphtheriae* biotype *mitis* (ATCC 27012 and CDC E8392), *C. diphtheriae* biotype *intermedius* (CDC D7920), *C. diphtheriae* biotype *gravis* (CDC E6651), *C. ulcerans* (CDC KC279), non-toxigenic *C. diphtheriae* biotype *mitis* (ATCC 27010), *C. minutissimum* (ATCC 23348), *C. striatum* (CDC F378), *C. jeikeium* (ATCC 43734), *C. xerosis* (CDC E8081), *R. equi* (ATCC 33701 and ATCC 10146), *Nocardia asteroides* (ATCC 7772), and *Nocardia brasiliensis* (ATCC 7771). The ATCC and CDC strains were obtained, respectively, from the American Type Culture Collection, Rockville, MD, USA, and the Centers for Disease Control and Prevention, Atlanta, GA, USA.

Differentiation of *C. diphtheriae* from non-diphtherial *corynebacteria* was based on the results of both standard biochemical tests [14–16] and a commercial kit (API Coryne System, bioMérieux, La-Balme-les-Grottes, France) [17]. Stock cultures were maintained as a suspension in 10% skim milk containing 25% glycerol at −20 °C. Microorganisms were previously cultured in a Columbia Agar Base (BBL, Sparks, USA) with 5% sheep blood for 24–48 h at 37 °C during all the phenotypic and genotypic procedures described in this study.

2.2. Toxigenicity tests

All *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* strains were evaluated for toxigenicity by Vero cell citotoxicity assay [18] and, in addition, by PCR for the *tox* gene via the DT primer set (PCR-DT) targeting fragment A of the diphtheria toxin [19].

2.3. PCR–dtxR assay

DNA was extracted by boiling a suspension composed of a loopful of freshly cultured bacteria in 500 μl sterile water for 10 min. The suspension was centrifuged at 13,000 rpm and 2 μl supernatant was used in the final reaction described below. The PCR reaction was performed in a 25 μl volume containing: 1× Taq polymerase buffer, 1.5 mM $MgCl_2$, 4 μM of each of the dtxR primers [6], 200 μM deoxynucleoside triphosphates, and 1.25 units of Taq DNA polymerase (all reagents were purchased from Gibco BRL, Rockville, USA). Thermocycling was performed on a Minicycler PTC – 100 Programmable Thermal Controller (MJ Research, Watertown, USA) using 35 cycles at 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 90 s. The reaction terminated with a final extension step of 72 °C for 10 min.

Specificity of the PCR assay for *C. diphtheriae* was analyzed by using four primer sets targeting the *dtxR* gene described elsewhere [6]. The dtxR1 primer set (dtxR 1F GGGACTACAACGCAACAAGAA; dtxR 1R CAACGGTTTGGCTAACTGTA; nucleotide positions −123 to −103 and 116 to 135, respectively) was selected for investigation with non-diphtherial isolates.

3. Results

All *C. diphtheriae* strains were PCR–dtxR positive via the four sets of dtxR primers. PCR–dtxR results confirmed the identification of the *C. diphtheriae* strains achieved by conventional biochemical tests and by the API Coryne System (Table 1). Identical PCR–dtxR patterns were found in all *C. diphtheriae* phenotypes, regardless of biotypes (*gravis*, *intermedius*, *mitis*, and *belfanti*), sucrose fermentation biotypes, toxigenicity and isolation sites, as exemplified in Fig. 1. Fifty-five non-toxigenic *C. diphtheriae* strains, including the ATCC 27010-type strain, all evaluated by the PCR-DT and Vero cell citotoxicity tests, were all positive for PCR–dtxR assay.

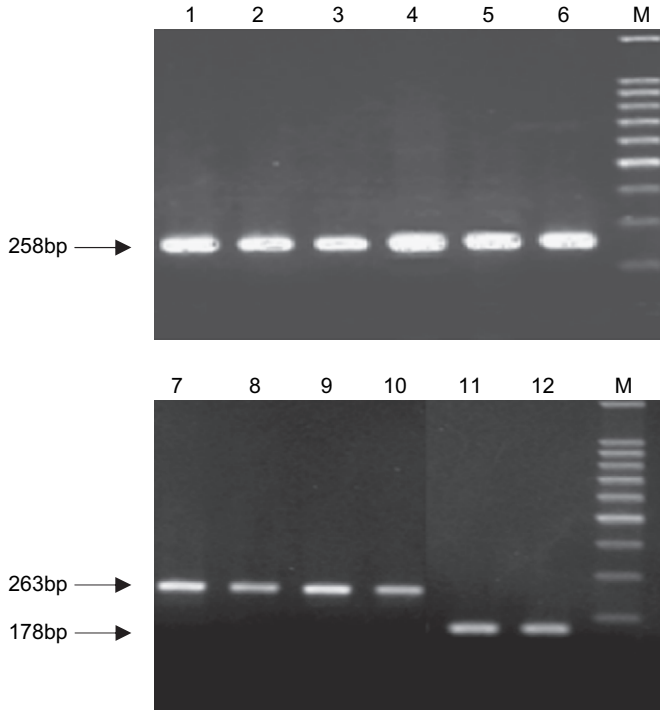

Fig. 1. Agarose gel electrophoresis of PCR products for identification of *Corynebacterium diphtheriae*. Control strains: lanes 1, 7, 9 and 11, non-toxigenic *C. diphtheriae* ATCC 27010 biotype *mitis*; lane 2, toxigenic *C. diphtheriae* ATCC 27012 biotype *mitis*. Clinical isolates: lane 3, toxigenic *C. diphtheriae* biotype *belfanti*; lane 4, non-toxigenic *C. diphtheriae* biotype *gravis*; lane 5, 8, 10 and 12, non-toxigenic *C. diphtheriae* biotype *mitis*; lane 6, toxigenic *C. diphtheriae* biotype *mitis*. Lanes 1–6, PCR products with primer set dtxR1 (258 bp); lanes 7 and 8, PCR products with primer set dtxR2 (263 bp); lanes 9 and 10, PCR products with primer set dtxR3 (262 bp); lanes 11 and 12, PCR products with primer set dtxR4 (178 bp). M, molecular size marker 100-bp ladder.