A multiplex PCR assay for simultaneous detection of *Corynebacterium diphtheriae* and differentiation between non-toxigenic and toxigenic isolates

The importance of identifying *Corynebacterium diphtheriae* can be appreciated since diphtheria remains endemic in many countries, alongside the risk of epidemic outbreaks and the existence of a large number of non-immunized people worldwide (Galazka & Robertson, 1995; Damasco *et al.*, 2005). The features of infections caused by *C. diphtheriae* have changed over the decades, and are most clearly emphasized by the emergence of non-toxigenic strains causing atypical diseases, such as endocarditis, septic arthritis or infection in unusual anatomical sites (Mattos-Guaraldi & Formiga, 1998; Efstratiou & George, 1999; Reacher *et al.*, 2000; Mattos-Guaraldi *et al.*, 2003). Rapid and reliable methods are needed for identifying a *C. diphtheriae* infection, thus aiding in appropriate and timely patient management, and improved monitoring of cases.

The use of multiplex PCR is becoming increasingly important in the diagnosis of infectious diseases, and could be a simple and fast alternative procedure for identification of *C. diphtheriae*, including for screening for non-toxin-gene- and toxin-gene-bearing strains (Cianciotto & Groman, 1997). In this study, a multiplex PCR assay was developed as a rapid and simple method for the identification of *C. diphtheriae*, and for the differentiation between non-toxigenic and toxigenic strains among non-pigmented Gram-positive rods.

The study was undertaken with 84 *C. diphtheriae* strains from the culture collection of the Diphtheria Laboratory, Universidade do Estado do Rio de Janeiro, Brazil, as follows: 74 strains of *C. diphtheriae* subsp. *mitis*, 5 of *C. diphtheriae* subsp. *gravis* and 5 of *C. diphtheriae* subsp. *belfanti*; 33 of the strains were toxigenic and 51 were non-toxigenic; 40 of the strains were non-sucrose fermenting and 44 were sucrose fermenting. These *C.*

diphtheriae strains were isolated from skin infections ($n=34$), blood-stream infections ($n=4$) and respiratory tract infections ($n=46$). This study also included three non-toxigenic *Corynebacterium ulcerans*, in addition to five non-toxigenic *Corynebacterium pseudotuberculosis* clinical strains kindly provided by Vasco Azevedo, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. *C. diphtheriae* reference strains from the American Type Culture Collection (ATCC), Rockville, MD, USA, and from Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, were also used in experiments: non-toxigenic *C. diphtheriae* subsp. *mitis* ATCC 27010, toxigenic *C. diphtheriae* subsp. *mitis* ATCC 27012, CDC E8392 and Park Williams 8, *C. diphtheriae* subsp. *intermedius* CDC D7920, *C. diphtheriae* subsp. *gravis* CDC E6651. Micro-organisms were identified by conventional biochemical methods (Mattos-Guaraldi & Formiga, 1998; Funke & Bernard, 2003), DNase test (Pimenta *et al.*, 2008b) and by the API Coryne system (bioMérieux) in accordance with the manufacturer's instructions.

Cytotoxicity of *C. diphtheriae* strains, including reference strains, was also evaluated by PCR using a primer pair targeted to a portion of fragment A of the *tox* gene (PCR-DTA, 258 bp), as well as by the 'gold-standard' Vero cells cytotoxicity assay (Efstratiou *et al.*, 1998). The multiplex PCR assay was performed using three primer pairs corresponding to *dtxR* gene (DtxR1F and DtxR1R, 258 bp) and fragments of portions A (Dipht 2F and Dipht 4R, 719 bp) and B (Dipht 6F and Dipht 7R, 534 bp) of the *tox* gene (Nakao *et al.*, 1996). Bacterial DNA was extracted by boiling a suspension composed of a loopful of freshly cultured bacteria in 500 µl sterile water for 10 min. The PCR was performed in a 25 µl volume containing: 1 × *Taq* polymerase buffer,

1.5 mM MgCl₂, 200 µM dNTPs, 4 µM each of the primers, 1.25 units *Taq* DNA polymerase (all reagents from Gibco-BRL) and 2 µl template DNA. Thermo cycling was performed using 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min. The reaction was concluded with a final extension step of 72 °C for 10 min.

Results of the multiplex PCR of *C. diphtheriae* strains are shown in Fig. 1. Despite differences in the sites of isolation, biotypes and subspecies, all 84 *C. diphtheriae* clinical and 6 control strains tested exhibited at least one amplification product with the expected size of 258 bp, corresponding to the *dtxR* gene. A recent investigation indicated the use of the *dtxR* gene as a species-specific marker for *C. diphtheriae* (Pimenta *et al.*, 2008a). In that work, results of the PCR-dtxR were 100 % positive for non-toxigenic and toxigenic *C. diphtheriae* strains. Conversely, the PCR-dtxR did not show an amplification signal from clinical isolates identified as non-diphtherial *Corynebacterium* species. In the present study, all three *C. ulcerans* and five *C. pseudotuberculosis* strains tested were negative for PCR-dtxR. PCR-dtxR completely correlated with the standard biochemical and commercial identification for all *C. diphtheriae* strains tested, indicating the feasibility of this reaction. In the present study, a complete agreement between results of multiplex PCR and PCR-dtxR was observed for all *C. diphtheriae* strains tested.

In addition to the *dtxR* gene amplicon, two amplification products of 719 and 534 bp corresponding to fragments of portions A and B of the *tox* gene, respectively, were obtained by multiplex PCR for all toxigenic strains tested. Results were 100 % concordant with those observed in PCR assays using the primers targeted to domains A or B in separate reactions (Pimenta *et al.*, 2008a). A total of 33 clinical and 5 control strains were

Fig. 1. Results of multiplex PCR for the identification of *C. diphtheriae* and the differentiation between non-toxicogenic and toxicogenic isolates. Lane 1, ATCC 27010; lanes 2 to 4, non-toxicogenic clinical isolates; lane 5, ATCC 27012; lane 6, Park Williams 8; lane 7, CDC E6651; lanes 8 to 11, toxicogenic clinical isolates. M, 100 bp DNA ladder.

identified as toxicogenic, and 51 clinical strains and ATCC 27010 as non-toxicogenic by multiplex PCR. Complete agreement between the results of multiplex PCR, PCR-DTA and Vero cell cytotoxicity assays was observed for all *C. diphtheriae* strains tested. Toxin-gene-bearing strains (Cianciotto & Groman, 1997) were not observed among the *C. diphtheriae* strains tested. All non-toxicogenic *C. ulcerans* and non-toxicogenic *C. pseudotuberculosis* clinical strains were negative for multiplex PCR and PCR-DTA. Since *C. ulcerans* and *C. pseudotuberculosis* are not commonly related to human and animal infections in Rio de Janeiro, Brazil, additional study remains necessary in order to investigate the use of multiplex PCR for *C. ulcerans* and *C. pseudotuberculosis* strains.

The identification of *C. diphtheriae* should be initiated without delay following the isolation of any suspicious colonies. Phenotypic identification procedures tend to be technically demanding, or lacking in sensitivity, or else have limited evaluations. In general, these methods require at least 2 days from the selection of a suspicious organism. Important advantages of multiplex PCR over conventional biochemical procedures are its rapidity, ease of performance, the large number of strains that can be simultaneously tested for and the fact that interpretation of a PCR multiplex assay is simple. Identification by PCR proved to be

reliable, and could be extremely important because it allows rapid application of control procedures and treatment of diphtheria or *C. diphtheriae* systemic infections. The multiplex PCR system may be incorporated into routine analyses in clinical diagnosis laboratories and/or epidemiological research.

Acknowledgements

This work was supported by CNPq, CAPES, FAPERJ, SR-2/UERJ and the Programa de Núcleo de Excelência (PRONEX) of the Brazilian Ministry of Science and Technology.

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