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Identification and molecular discrimination of toxigenic and nontoxigenic diphtheria *Corynebacterium* strains by combined real-time polymerase chain reaction assays ☆

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ABSTRACT

With the recognition of several diphtheria outbreaks and the emergence of nontoxigenic corynebacteria strains, there has been renewed interest in the development of laboratory diagnostic methods. Previously reported polymerase chain reaction (PCR) assays can have low diagnostic sensitivity or give species misidentifications among clinical isolates. The aim of the present study was the development of combined real-time PCR assays, based on the *tox* and *rpo*B genes, for the detection and differentiation of toxigenic and nontoxigenic corynebacteria. By the PCR tox assay, it was possible to perform the direct identification of DT *tox* gene of *Corynebacterium diphtheriae* and *Corynebacterium ulcerans*, while the PCR *rpo*B assay differentiated *C. diphtheriae* from *C. ulcerans*, irrespective of their toxigenic status. In addition, we detected the DT toxin of *Corynebacterium pseudotuberculosis* for the first time. These assays revealed high sensitivity, specificity, and reproducibility, and the availability of plasmid controls will facilitate further research into the diagnostics of diphtheria corynebacteria.

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1. Introduction

Diphtheria caused by *Corynebacteriun diphtheriae* is a well-controlled disease in industrialized countries, thanks to vaccination against diphtheria toxin. However, thousands of deaths can occur if there is a breach of the preventive measures, such as vaccination of children and booster policy of adults, as learned by the sudden, unexpected, and severe epidemic that occurred in the mid 1990s in Russia and in the New Independent States of the former Soviet Union (Dittmann et al., 2000; Galazka, 2000). Diphtheria cases are still reported, after the big epidemic wave in Latvia, Russia, and Ukraine. Furthermore, diphtheria is still endemic in several parts of the world, particularly in Asia (India, Indonesia, Iran, Nepal, Pakistan, etc.), Africa (Ghana), and South America (Brazil) (Sing et al., 2005; Wagner et al., 2010; World Health Organization).

Diphtheria is caused by toxin-producing corynebacteria. Not only *C. diphtheriae*, but also *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* have the potential to produce diphtheria toxin (DT) and hence can cause classic respiratory diphtheria as well as cutaneous diphtheria (Efstratiou and Maple, 1994). It is noteworthy that, in recent years, *C. ulcerans* infections have been reported worldwide and fatal infections have occurred (Bonmarin et al., 2009; Elden et al., 2007; Tiwari et al., 2008; von Hunolstein et al., 1999). *C. diphtheriae* strains, whether toxigenic or nontoxigenic, have been found to cause severe and often fatal systemic infections also in immunized subjects (De Winter et al., 2005) (Funke et al., 1999) (Gubler et al., 1998; Patey et al., 1997; Puliti et al., 2006; Reacher et al., 2000; Romney et al., 2006; von Hunolstein et al., 2003). As the mortality of infections caused by the 3 corynebacteria species mentioned above is almost entirely due to DT, the availability of rapid methods for the identification of the species as well as for the detection of DT *tox* gene is of primary importance. DTs produced by the different corynebacteria are immunologically identical (Lipsky et al., 1982), although the DT of *C. diphtheriae* and that of *C. ulcerans* differ at the nucleotide and amino acid levels (95.4% homology), while the sequence of *C. pseudotuberculosis* DT is not known (Sing et al., 2005),(Nakao et al., 1997; Schuhegger et al., 2008a; Sing et al., 2003).

The identification and characterization of suspected corynebacteria isolates are carried out by phenotypic methods (Efstratiou and George, 1999; Engler et al., 1997; Neal and Efstratiou, 2009), as well as by molecular techniques including classical or real-time polymerase chain reaction (PCR) detection of the *tox* gene (Mothershed

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et al., 2002; Pallen et al., 1994; Schuhegger et al., 2008b; Sing et al., 2011), RNA polymerase beta subunit-encoding gene (*rpo*B) sequencing (Khamis et al., 2004), and, more recently, also by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Konrad et al., 2010). Real-time PCR is a fast and reliable tool for the identification of putative toxigenic corynebacteria. So far, 3 different real-time PCR assays have been described for laboratory diagnosis, all based on the recognition of DT *tox* gene (Mothershed et al., 2002; Schuhegger et al., 2008b; Sing et al., 2011). Two methods employ TaqMan hydrolysis probes (Mothershed et al., 2002; Schuhegger et al., 2008b), while the system of Sing et al. (Sing et al., 2011) was developed with LightCycler hybridization probes (Roche Diagnostics, Mannheim, Germany) that allow the simultaneous detection and differentiation of *tox* genes from *C. diphtheriae* and *C. ulcerans*. Two of these methods (Mothershed et al., 2002; Sing et al., 2011) have some limitations such as they miss the detection of atypical *C. ulcerans* strains (Schuhegger et al., 2008a; Cassiday et al., 2008) or misclassify a small number of *C. diphtheriae* strains (Sing et al., 2011).

Problems encountered using real-time PCR during the recent years, determined by the *heterogeneity* of *tox* genes (Schuhegger et al., 2008a; Sing et al., 2003) and the reemergence of toxigenic and nontoxigenic corynebacteria (Puliti et al., 2006; Reacher et al., 2000; Romney et al., 2006), advocate for an improvement in diagnostic skills. In order to support fast clinical decisions in the management of a critical patient, an accurate species differentiation is needed not only to distinguish toxigenic corynebacteria but also to identify pathogenic nontoxigenic corynebacteria.

To this aim, we describe and evaluate a combination of 2 different real-time PCR assays based on the *tox* and *rpo*B genes using fluorescence resonance energy transfer (FRET) probes and melting curve analyses for a rapid identification of toxigenic and nontoxigenic strains and for differentiation of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*.

2. Materials and methods

2.1. Bacterial strains and simulated clinical samples

A total of 79 strains either belonging to the collection of the Istituto Superiore di Sanità (Italian Public Health Institute), or kindly provided by the Health Protection Agency (UK), were included in this study: 22 *tox*-bearing strains (8 *C. diphtheriae* biotype *mitis*, 7 *C. diphtheriae* biotype *gravis*, 1 *C. diphtheriae* biotype *intermedius*, 5 *C. ulcerans*, and 1 *C. pseudotuberculosis*) and 16 nontoxigenic strains (4 *C. diphtheriae* biotype *mitis*, 7 *C. diphtheriae* biotype *gravis*, 4 *C. diphtheriae* biotype *belfanti*, 1 *C. ulcerans*) were examined (Table 1). Additionally, 13 DT-negative strains of various *Corynebacterium* spp. and 28 strains

Table 1
Toxigenic and nontoxigenic corynebacteria strains, used to evaluate the sensitivity and specificity of real-time PCR assays.

Isolate	Species	Biotype[a]	Elek test	tox PCR[b]	Real-time PCR assays, +/− (Tm)[c]	
					PAC (*tox*)	*rpo*B
6918	*C. diphtheriae*	M	−	+	+ (63.0)	+ (70.0)
6919	*C. diphtheriae*	G	+	+	+ (63.0)	+ (70.0)
6920	*C. diphtheriae*	B	−	−	−	+ (70.0)
6922	*C. diphtheriae*	I	−	+	+ (63.0)	+ (70.0)
H101390001	*C. diphtheriae*	G	−	−	−	+ (70.0)
H095340738	*C. diphtheriae*	M	+	+	+ (63.0)	+ (70.0)
H101160113	*C. diphtheriae*	B	−	−	−	+ (70.0)
4112	*C. diphtheriae*	M	−	−	−	+ (70.0)
2834	*C. diphtheriae*	M	−	−	−	+ (70.0)
2992	*C. diphtheriae*	M	−	−	−	+ (70.0)
3319	*C. diphtheriae*	M	−	−	−	+ (70.0)
4395	*C. diphtheriae*	G	−	−	−	+ (70.0)
4366	*C. diphtheriae*	G	−	−	−	+ (70.0)
4401	*C. diphtheriae*	G	−	−	−	+ (70.0)
4603	*C. diphtheriae*	G	−	−	−	+ (70.0)
2989	*C. diphtheriae*	G	−	−	−	+ (70.0)
2767	*C. diphtheriae*	G	+	+	+ (63.0)	+ (70.0)
C7 (B197)	*C. diphtheriae*	M	−	+	+ (63.0)	+ (70.0)
NCTC 3984	*C. diphtheriae*	G	Weakly positive	+	+ (63.0)	+ (70.0)
NCTC 10356	*C. diphtheriae*	B	−	−	−	+ (70.0)
NCTC 10648	*C. diphtheriae*	G	+	+	+ (63.0)	+ (70.0)
2912	*C. diphtheriae*	M	+	+	+ (63.0)	+ (70.0)
4252	*C. diphtheriae*	B	−	−	−	+ (70.0)
4505	*C. diphtheriae*	G	+	+	+ (63.0)	+ (70.0)
4506	*C. diphtheriae*	M	+	+	+ (63.0)	+ (70.0)
4507	*C. diphtheriae*	G	+	+	+ (63.0)	+ (70.0)
4508	*C. diphtheriae*	M	+	+	+ (63.0)	+ (70.0)
4509	*C. diphtheriae*	M	+	+	+ (63.0)	+ (70.0)
4510	*C. diphtheriae*	M	+	+	+ (63.0)	+ (70.0)
4511	*C. diphtheriae*	G	+	+	+ (63.0)	+ (70.0)
4604	*C. diphtheriae*	G	−	−	−	+ (70.0)
6921	*C. ulcerans*		Weakly positive	+	+ (60.5)	+ (55.5)
H094920187	*C. ulcerans*		Weakly positive	+	+ (60.5)	+ (55.5)
5338	*C. ulcerans*		+	+	+ (60.5)	+ (55.5)
NCTC 12077	*C. ulcerans*		−	−	−	+ (59.5)
3003	*C. ulcerans*		+	+	+ (63.0)	+ (55.5)
3910	*C. ulcerans*		+	+	+ (60.5)	+ (55.5)
CD99-126	*C. pseudotuberculosis*		+	+	+ (60.5)	−

Unexpected Tm's are underlined.
[a] Biotype: M = mitis; G = gravis; B = belfanti; I = intermedius.
[b] Toxigenicity was determined by the Elek test and the presence of tox gene was performed by a conventional and real-time PCR as previously described in Materials and Methods.
[c] Tm = Melting temperature (°C).