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Detection and Characterization of Diphtheria Toxin Gene-Bearing *Corynebacterium* Species through a New Real-Time PCR Assay

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ABSTRACT Respiratory diphtheria, characterized by a firmly adherent pseudomembrane, is caused by toxin-producing strains of *Corynebacterium diphtheriae*, with similar illness produced occasionally by toxigenic *Corynebacterium ulcerans* or, rarely, *Corynebacterium pseudotuberculosis*. While diphtheria laboratory confirmation requires culture methods to determine toxigenicity, real-time PCR (RT-PCR) provides a faster method to detect the toxin gene (*tox*). Nontoxigenic *tox*-bearing (NTTB) *Corynebacterium* isolates have been described, but impact of these isolates on the accuracy of molecular diagnostics is not well characterized. Here, we describe a new triplex RT-PCR assay to detect *tox* and distinguish *C. diphtheriae* from the closely related species *C. ulcerans* and *C. pseudotuberculosis*. Analytical sensitivity and specificity of the assay were assessed in comparison to culture using 690 previously characterized microbial isolates. The new triplex assay characterized *Corynebacterium* isolates accurately, with 100% analytical sensitivity for all targets. Analytical specificity with isolates was 94.1%, 100%, and 99.5% for *tox*, Diph_*rpoB*, and CUP_*rpoB* targets, respectively. Twenty-nine NTTB *Corynebacterium* isolates, representing 5.9% of 494 nontoxigenic isolates tested, were detected by RT-PCR. Whole-genome sequencing of NTTB isolates revealed varied mutations putatively underlying their lack of toxin production, as well as eight isolates with no mutation in *tox* or the promoter region. This new *Corynebacterium* RT-PCR method provides a rapid tool to screen isolates and identify probable diphtheria cases directly from specimens. However, the sporadic occurrence of NTTB isolates reinforces the viewpoint that diphtheria culture diagnostics continue to provide the most accurate case confirmation.

KEYWORDS *Corynebacterium*, diphtheria, diphtheria toxin, diphtheriae, real-time PCR, ulcerans

Diphtheria is a vaccine preventable respiratory disease that, despite available clinical treatment, has a case fatality rate of approximately 10% (1). Infection caused by *Corynebacterium diphtheriae* is marked by a firmly adherent throat pseudomembrane and, in some severe disease, a swollen neck ("bull neck"). Severe symptoms are typically mediated by an exotoxin produced by *C. diphtheriae* strains that are infected by a lysogenic bacteriophage carrying the diphtheria toxin gene (*tox*) (2). The related species *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* may also be toxigenic and produce diphtheria-like illness in humans, though they are thought to primarily infect domesticated animals (3). In the United States, respiratory diphtheria is rare and is travel associated (4). However, toxigenic *C. diphtheriae* continues to circulate, leading to disease re-emergence when vaccination gaps occur, as seen recently with

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outbreaks in South Africa ($n = 21$ confirmed cases), India ($n = 533$ confirmed cases), and Bangladesh ($n = 271$ confirmed cases) and endemic infection in Haiti ($n > 350$) (5–9). Diagnostic capacity is important to maintain because of the potential for disease re-emergence domestically and internationally.

Nontoxigenic *C. diphtheriae* and *C. ulcerans* have been increasingly identified in respiratory and nonrespiratory infections, most likely related to the adoption of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry as a clinical diagnostic tool (10–13). Nontoxigenic *C. diphtheriae* biotype belfanti is commonly isolated from patients with prolonged sinus infections (14). Although this biotype has been proposed recently as a discrete species named *Corynebacterium belfantii* (15), traditional *C. diphtheriae* biotype belfanti nomenclature is used here. While cutaneous and other nonrespiratory infections may be caused by nontoxigenic *C. diphtheriae* and *C. ulcerans*, occasionally, toxigenic *C. diphtheriae* or *C. ulcerans* is isolated from these infections, representing a potential risk for transmission to result in respiratory diphtheria (10, 16, 17).

Laboratory confirmation of diphtheria requires culture isolation and toxigenicity determined by the Elek test, which detects an immunoprecipitation reaction between toxin and diphtheria antitoxin (DAT) (18). However, few laboratories maintain this testing capacity because of low demand and worldwide shortage of DAT (WHO website, accessed 7 January 2020 [http://www.who.int/immunization/sage/meetings/2017/april/3_Diphtheria_anti_toxin.pdf]). Currently in the United States, only the Pertussis and Diphtheria Laboratory at the Centers for Disease Control and Prevention (CDC) determines toxigenicity in *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* isolates.

Culture identification and toxigenicity determination typically require 2 to 5 days, by which time a clinician may have already started DAT treatment for a suspected respiratory diphtheria case. As a supplement to culture diagnostics, molecular assays provide rapid and sensitive screening that improves the speed of reporting. Previously, conventional PCR detection of *tox* and the diphtheria toxin repressor gene (*dtxR*) was developed (19). In addition, a real-time PCR (RT-PCR) assay to detect *tox* coding regions for toxin subunits A and B was previously developed and performed at CDC, allowing rapid detection within 24 to 36 h of specimen collection (20). Although the assay demonstrated 100% sensitivity and specificity in the initial examination, *tox* mutations in additional isolates yielded false-negative results. In one study, 7/11 toxigenic *C. ulcerans* isolates were weakly detected with the *toxA* target and were not detected with the *toxB* target (21). In the same year, a single-target RT-PCR assay was published that detected *C. ulcerans* and *C. diphtheriae tox* (22). Additional available diphtheria RT-PCR assays include one that detects and distinguishes *C. diphtheriae* and *C. ulcerans tox* on the Roche LightCycler and Applied Biosystem 7500 platforms (23). More recently, Public Health England developed a quadruplex assay that detects *tox*, identifies *C. diphtheriae*, and detects *C. ulcerans*/*C. pseudotuberculosis* (24). A fourth target detects green fluorescent protein (GFP) DNA as an internal PCR control. This assay was also recently updated by replacing GFP DNA with a 16S rRNA gene primer set to provide a quality check of the template extract (25).

To improve analytical sensitivity, we developed a three-target *Corynebacterium* triplex RT-PCR assay that rapidly detects diphtheria *tox* and identifies *C. diphtheriae* and the closely related species *C. ulcerans* and *C. pseudotuberculosis*. When clinical specimens are tested with the *Corynebacterium* triplex assay, a separate RT-PCR targeting the human RNase P gene is included to ensure specimen and DNA extract quality. Nontoxigenic *tox*-bearing (NTTB) isolates of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* have also been found in the United States and Europe, with various mutations that prevent toxin production (25–28), potentially confounding RT-PCR interpretation if toxigenicity is not confirmed in an isolate with the Elek test. The assay provides a rapid screening tool to identify potential toxigenic isolates requiring confirmatory Elek testing, while isolates not bearing *tox* may be reported quickly to the submitting laboratory.

Analytical validation of the CDC *Corynebacterium* triplex RT-PCR assay was performed by testing isolates from the CDC culture collection and recent clinical specimens. NTTB *Corynebacterium* isolates were characterized through genome sequence analysis to determine mutations that confer lack of toxigenicity.

MATERIALS AND METHODS

This project was reviewed in accordance with CDC human research protection procedures and was determined to be research not involving human subjects; therefore, institutional review board approval was not required.

Clinical specimens and bacterial strains. Clinical specimens from suspected diphtheria cases were sent routinely to CDC from domestic and international sources for diphtheria culture and/or RT-PCR diagnostic testing, according to guidelines published on the CDC website (https://www.cdc.gov/diphtheria/laboratory.html). All specimens collected in the years 2015 to 2017 were included in this study ($n = 105$), the majority of which were throat swabs ($n = 91$).

A convenience sample of clinical isolates of *Corynebacterium* and other species was selected from the CDC collection. *C. diphtheriae* CD001 (NCTC 10648) and *C. ulcerans* CD075, both toxigenic, served as RT-PCR positive controls. In total, 690 microbial isolates were tested, including *C. diphtheriae* ($n = 373$), *C. ulcerans* ($n = 141$), *C. pseudotuberculosis* ($n = 28$), other *Corynebacterium* species ($n = 28$), and other respiratory and nonrespiratory pathogenic microorganisms ($n = 120$). *Corynebacterium* species isolates were collected from humans and other mammals in the years 1948 to 2018, from 42 U.S. states and territories and 24 additional countries. Isolate sources included respiratory and nonrespiratory sites. Forty-eight isolates were toxigenic, including 32 *C. diphtheriae* and 16 *C. ulcerans* isolates. Data Set S1 contains the list of non-*Corynebacterium* species that were tested.

Bacterial culture conditions and isolate characterization. Bacterial strains were stored on cryobeads (Microbank, Pro-Lab Diagnostics, Round Rock, TX) at −70°C and were grown on tryptic soy agar (TSA) with 5% sheep blood at 37°C overnight (16 to 24 h). As a lipophilic corynebacterium, *C. diphtheriae* biotype intermedius could exhibit slower growth than other biotypes. Species identification and *C. diphtheriae* biotype were determined with Gram stain and the API Coryne biochemical test strip (bioMérieux, Durham, NC), following manufacturer instructions. Results were interpreted with the API test database. Rarely, 16S rRNA gene sequencing was performed if needed to resolve ambiguous API test results. Toxigenicity of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* isolates was determined by the Elek test (18).

DNA extraction. (i) Control strains. DNA was extracted using a modified method for the QIAamp DNA minikit (catalog no. 51106; Qiagen, Germantown, MD). Bacterial growth from overnight cultures of CD001 (NCTC 10648) and CD075 was suspended in 180 μl of sterile Tris-EDTA (TE) buffer in 1.5 ml Eppendorf tubes. The suspension was vortexed and then incubated at 99°C for 30 min with constant shaking on a heat block. Suspensions were treated with lysozyme solution (10 mg/ml) at 37°C for 30 min with constant shaking; then, 25 μl of proteinase K and 200 μl of buffer AL from the Qiagen kit were added. The solution was mixed by vortexing and incubated on the heat block for 2 h at 70°C and then for an additional 30 min at 95°C. From here, DNA purification proceeded according to the Qiagen kit protocol, with the additional incubation of columns and elution buffer at 70°C for 5 min before elution of DNA from the columns. Eluted DNA was quantified in a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA) and diluted to 20 ng/μl per strain. Positive-control strains were diluted to 10^6 genome equivalents per 2 μl (0.5×10^6 per μl), either in single format or with two strains pooled.

(ii) Isolates. Bacterial growth from overnight agar plates was suspended in 1 ml of 0.85% saline, vortexed, and incubated at 99°C for 30 min. Suspensions were centrifuged at a relative centrifugal force (rcf) of 18,500 for 5 min at room temperature, and the supernatant was transferred to a new tube for storage at 2 to 8°C until tested by RT-PCR. DNA concentration was measured in a Nanodrop 2000 spectrophotometer in extracts from bacterial isolates that were expected to be negative by the *Corynebacterium* RT-PCR assay. Alternatively, total nucleic acid (TNA) was extracted from 200 μl of an isolate colony suspended in 5 ml phosphate-buffered saline (PBS) buffer and eluted into 100 μl using the MagNA Pure Compact instrument with total nucleic acid isolation kit (Roche Applied Science, IN, USA) following the manufacturer's instructions. The TNA was normalized to 3 ng/μl using the Agilent Bioanalyzer.

(iii) Specimens. DNA was extracted from specimens using a QIAamp DNA minikit with modifications to the protocol described for positive-control strains, except that the first incubation (99°C, 30 min) was omitted (20). DNA was stored at 2 to 8°C and analyzed within 48 h by *toxAB* real-time PCR (described below). After analysis, DNA was stored frozen until reanalyzed by *Corynebacterium* triplex RT-PCR (described below).

***Corynebacterium toxAB* real-time PCR.** Singleplex RT-PCR targeting diphtheria toxin subunits A and B was performed as described (20). Amplification was performed in 20-μl reaction mixtures containing 10 μl Quanta PerfeCTa qPCR Toughmix with uracil *N*-glycosylase (UNG) and low carboxy-X-rhodamine (ROX) (Quanta BioSciences, Inc., Gaithersburg, MD), 1 μM (each) forward and reverse primer, 0.1 μM probe, and 2 μl template DNA. Reaction mixtures were subjected to amplification and detection in an Applied Biosystems 7500 fastDx instrument (Life Technologies Corp., Carlsbad, CA) by preincubation at 50°C for 2 min, denaturation at 95°C for 10 min, and then 45 cycles of 95°C for 5 s and 60°C for 30 s. The cycle threshold (C_T) was set between 0.02 and 0.2, and a C_T value of <40 was positive.

***Corynebacterium* triplex real-time PCR.** Primers and TaqMan probes were designed for specific detection of *C. diphtheriae rpoB* (Diph_*rpoB*), *C. ulcerans* or *C. pseudotuberculosis rpoB* (CUP_*rpoB*), and the

TABLE 1 *Corynebacterium* triplex and RNase P RT-PCR primers and probes[a]

Target gene	Primer or probe name	Sequence (5′-3′)	Position (bp)[b]	Amplicon size (bp)	Concn (μM)
tox	Coryne_toxF	GGCCAAGATGCGATGTATG	189573–189591	100	0.5
	Coryne_toxR	CCCAATCAAGATTTATGCATGAC	189650–189672		0.5
	Coryne_toxP-FAM	FAM-TCGTGTCAGGCGATCAGTAGGTAGC-BHQ1	189620–189644		0.1
C. diphtheriae rpoB (Diph_*rpoB*)	Diph_rpoBF	CGCCAGCAAGAAGAGCT	409927–409943	120	0.5
	Diph_rpoBR	AGGCTCAGAAAGAGACAGC	410028–410046		0.5
	Diph_rpoBP-HEX	HEX-CGACTCGGTTCGCGTAACAAGCG-BHQ1	409947–409969		0.1
C. ulcerans/*C. pseudotuberculosis rpoB* (CUP_*rpoB*)	CUP_rpoBF	TAGATTCCTTCGCATGGCTCA	405526–405546	135	1
	CUP_rpoBR	CGGAATAATCCTGAATCGGAG	405640–405660		1
	CUP_rpoBP-Q670	Q670-CAGGAGGAGCTRGGTGAAARCGTCC-BHQ3	405576–405600		0.2
Human RNase P gene (for specimens only)[c]	RNaseP-F	CCAAGTGTGAGGGCTGAAAAG	Not applicable	80	0.4
	RNaseP-R	TGTTGTGGCTGATGAACTATAAAAGG			0.4
	RNaseP-P	FAM-CCCCAGTCTCTGTCAGCACTCCCTTC-BHQ1			0.1

[a]FAM, fluorescein; HEX, hexachlorofluorescein; Q670, Quasar 670; BHQ, black hole quencher.
[b]Position is based on *C. diphtheriae* NCTC 13129 (NC_002935) for *tox* and *C. diphtheriae rpoB* target sites; *C. ulcerans* 809 (NC_017317) was the reference for the CUP_*rpoB* primer/probe set.
[c]From reference 30.

Corynebacterium toxin gene (*tox*). Oligonucleotides were designed manually through sequence alignment of *rpoB* (GenBank accession numbers CP003217, CP010795, and AB828262) or *tox* (AY820132, FJ858272, and JN176077) for the three species using the Clustal Omega web server (https://www.ebi.ac.uk/Tools/msa/clustalo/). The oligonucleotides were designed to optimize melting temperatures (T_m) and minimize intra- and intermolecular interactions. The specificity and inclusivity of each set of oligonucleotides were assessed by sequence comparison to the National Center for Biotechnology Information (NCBI) nr database using Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/) (29). The *Corynebacterium tox*, *C. diphtheriae rpoB*, and *C. ulcerans*/*C. pseudotuberculosis rpoB* oligonucleotide sets were assessed relative to one another to ensure no intermolecular interactions or dimerization potential that could yield a false-positive amplification signal. Sequence comparison by BLASTn alignment was also used to ensure specificity for the oligonucleotide sets when combined in a single reaction mix. Primers and probes were synthesized in the Biotechnology Core Facility Branch at CDC. Primer and probe sequences, with reporter and quencher dyes, are listed in Table 1.

Each reaction was performed in a 20-μl volume that included 10 μl Quanta qScript RT Toughmix with low ROX or PerfeCTa qPCR Toughmix with UNG and low ROX(Quanta BioSciences, Inc., Gaithersburg, MD), 2 μl template, and primer and probe concentrations as described in Table 1. A well with water as a no-template control (NTC) was included between specimens on the PCR plate. Reactions were amplified and detected with an Applied Biosystems 7500 fastDx (Life Technologies Corp., Carlsbad, CA). The threshold was set at 0.2, and C_T values less than 40 were considered positive for each target. Specimens with positive *tox* gene results were interpreted as positive for *tox*-bearing *C. diphtheriae* or *C. ulcerans*/*C. pseudotuberculosis* if either *rpoB* target was positive. Specimens with positive *tox* gene and negative *rpoB* target results were interpreted as *tox*-bearing *Corynebacterium* spp. Similarly, specimens positive for either *rpoB* target and negative for *tox* were designated *tox*-negative *C. diphtheriae* or *C. ulcerans*/*C. pseudotuberculosis*.

An RNase P assay was included as a separate external control to test clinical specimens for the presence of human DNA, as previously described, with the volume adjusted to 2 μl template in 20-μl reaction mixtures (30).

Calculations and comparisons. The *tox*, Diph_*rpoB*, and CUP_*rpoB* assays were tested empirically to evaluate analytical sensitivity and specificity, both individually and combined in a single reaction mix (triplex). The RT-PCR amplification efficiency of each reaction was determined by the formula $10^{[-1/\text{slope}]} - 1$ (31), and efficiencies were compared between single and triplex reactions to ensure no drop in efficiency. The limit of detection was determined with a dilution series tested in triplicate by three operators on 3 days and was calculated as genome equivalents of *C. diphtheriae* NCTC 13129 and *C. ulcerans* 809. Specificity was assessed initially by testing human nucleic acid, nuclease-free water for the no-template control, and isolates from the *Corynebacterium* genus excluding *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*. Additionally, the multiplex assay was screened against an extensive panel of respiratory and nonrespiratory microorganisms (Data Set S1). Inclusivity was evaluated by testing isolates of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*. Results for bacterial isolates were qualitatively compared to *toxAB* RT-PCR results and culture characteristics. Similarly, clinical specimen results were compared to *toxAB* RT-PCR and culture results, when available.

Whole-genome sequencing and analyses. Isolates that were determined to carry *tox* but did not produce diphtheria toxin in the Elek test (NTTB) were characterized by genome sequencing. Approximately 1×10^9 bacterial cells were pretreated with 5 mg/ml lysozyme at 37°C for 45 min with constant mixing at 500 rpm before genomic DNA extraction using the whole-blood DNA kit in a Maxwell rapid sample concentrator (RSC) (Promega Corporation, Madison, WI). Sequencing libraries were prepared with

TABLE 2 Isolates tested with *Corynebacterium* triplex RT-PCR[a]

Culture identity and toxigenicity				Current RT-PCR assay result			Previous assay result[b]	
Category and species	**Biotype**	**No. of isolates**	**Toxigenicity**	***tox***	**Diph_*rpoB***	**CUP_*rpoB***	***toxA***	***toxB***
Target *Corynebacterium* species								
C. diphtheriae	belfanti	84	−	−	+	−	−	−
		1[c]	−	+	+	−	+	+
		1	+	+	+	−	+	+
	gravis	146	−	−	+	−	−	−
		7	+	+	+	−	+	+
	intermedius	7	−	−	+	−	−	−
		5[c]	−	+	+	−	+	+
		1	+	+	+	−	+	+
	mitis	78	−	−	+	−	−	−
		15[c]	−	+	+	−	+	+
		23	+	+	+	−	+	+
	"felis" [d]	4[c]	−	+	+	−	+	−
C. diphtheriae	Not determined	1	−	−	+	−	−	−
C. pseudotuberculosis	NA	28	−	−	−	+	−	−
C. ulcerans	NA	121	−	−	−	+	−	−
		4[c]	−	+	−	+	−	−
		5	+	+	−	+	−	−
		8	+	+	−	+	+	−
		3	+	+	−	+	+	+
Total positives		542	48	77	373	169	68	56
Nontarget *Corynebacterium* species								
C. afermentans/C. coyleae	NA	1	NT	−	−	−	−	−
C. jeikeium	NA	1	NT	−	−	−	NT	NT
C. minutissimum	NA	1	NT	−	−	−	NT	NT
C. pseudodiphtheriticum	NA	14	NT	−	−	−	−	−
C. renale[e]	NA	3	−	−	−	+ (2/3)	−	−
C. striatum	NA	4	−	−	−	−	−	−
C. urealyticum	NA	1	−	−	−	−	−	−
Corynebacterium spp.	NA	3	−	−	−	−	−	−
Total		28	NA	0	0	2	0	0
Additional respiratory and nonrespiratory organisms								
Other bacterial pathogens	NA	99	NT	−	−	−	NT	NT
Yeast	NA	2	NT	−	−	−	NT	NT
Viruses	NA	19	NT	−	−	−	NT	NT
Total		120	0	0	0	0	0	0

[a]Results were compared to the previous *toxAB* RT-PCR assay and culture toxigenicity data by Elek. Species and *C. diphtheriae* biotype were determined by the API Coryne test. Diph_*rpoB*, *C. diphtheriae rpoB*; CUP, *C. ulcerans/C. pseudotuberculosis*; NA, not applicable; NT, not tested.
[b]From reference 20.
[c]Nontoxigenic *tox*-bearing isolate(s) ($n = 29$).
[d]"felis" is an informal strain designation suggested by Hall et al. (28).
[e]Two *C. renale* isolates were detected by CUP_*rpoB* with a C_T value of >32.

DNA extracts using the NEB Ultra library prep kit (New England Biolabs; Ipswich, MA). Shotgun sequencing was performed on a MiSeq sequencer (Illumina; San Diego, CA) using a 250-bp paired-end format. Raw sequencing reads were quality trimmed and filtered using cutadapt (v1.9) (32) and then *de novo* assembled with SPAdes (v3.9) (33). Mutations to *tox* and *toxP* were determined through comparison to toxigenic alleles by alignment with BLASTn or read mapping with snippy (v4.3.8) (https://github.com/tseemann/snippy).

Data availability. The raw sequence data are available from the NCBI Sequence Read Archive, organized under BioProject no. PRJNA541849.

RESULTS

Analytical sensitivity, specificity, and amplification efficiency. All three RT-PCR targets demonstrated 100% sensitivity for isolates, compared to the gold standards of species identification and toxigenicity determination with the Elek test (Table 2). All *C. diphtheriae* isolates were detected by Diph_*rpoB* and negative with CUP_*rpoB* ($n = 373$). Similarly, *C. pseudotuberculosis* and *C. ulcerans* isolates were negative with Diph_*rpoB* and positive with CUP_*rpoB* ($n = 169$). All toxigenic isolates were *tox* positive, including

TABLE 3 Mutations to *tox* in *C. diphtheriae* and *C. ulcerans* nontoxigenic *tox*-bearing isolates

Mutation to *tox*	Species and biotype (if applicable)	No. of isolates	Isolate(s)
Position 52, deletion within poly(G) tract[a]	*C. diphtheriae* mitis	3	PC0104, PC0105, PC0351
	C. diphtheriae "felis"[b]	4	PC0226, PC0229, PC0230, PC0231
Position 56, deletion within poly(C) tract	*C. diphtheriae* mitis	1	PC0153
Position 107, IS*1132* insertion	*C. diphtheriae* belfanti	1	PC0110
Position 331, nonsynonymous C to T	*C. diphtheriae* mitis	2	PC0381, PC0598
Position 797, deletion within poly A tract	*C. diphtheriae* mitis	9	PC0112, PC0113, PC0114, PC0115, PC0116, PC0117, PC0118, PC0119, PC0120
	C. diphtheriae intermedius	1	PC0155
No mutation in *tox* or promoter	*C. diphtheriae* intermedius	4	PC0132, PC0133, PC0134, PC0135
	C. ulcerans	4	PC0090, PC0190, PC0365, PC0640
Total		29	

[a]Identified in reference 26.
[b]"felis" is an informal strain designation suggested by Hall et al. (28).

five toxigenic *C. ulcerans* isolates that were not detected with the previous *toxAB* assay ($n = 48$). One hundred forty-six nontarget isolates were negative for all three targets (Table 2). Two *Corynebacterium renale* isolates were detected by CUP_*rpoB* with a C_T value of >32. In contrast, the mean C_T value of CUP_*rpoB* target detection in *C. ulcerans* and *C. pseudotuberculosis* isolates was 24.4 (standard deviation [SD], 3.3). The identities of the two isolates were confirmed with 16S rRNA gene sequencing, resulting in 99% similarity to *C. renale* in a BLAST query of the NCBI nr database (data not shown). Diph-*rpoB* and CUP_*rpoB* displayed 100% and 99.5% specificity, respectively. Equivalent results were obtained when the *Corynebacterium* triplex RT-PCR assay was tested in single and multiplex reactions (data not shown).

The lower limits of detection were 10 genome equivalents for *tox* and 100 genome equivalents for Diph_*rpoB* and CUP_*rpoB*, determined by testing pooled DNA from positive-control strains CD001 and CD075. Efficiency of amplification for the *Corynebacterium* triplex RT-PCR assay was also tested with positive-control cultures CD001 and CD075. The *tox* reaction demonstrated 103% and 98.4% efficiency for CD001 and CD075, respectively. Diph_*rpoB* and CUP_*rpoB* targets were 88.2% and 93.1% efficient, respectively. In all cases, R^2 was >0.999.

NTTB strains. Twenty-nine isolates were NTTB, including 25 *C. diphtheriae* and four *C. ulcerans* isolates (Table 2). The presence of NTTB isolates reduced *tox* target specificity to 94.1%. NTTB *C. ulcerans* isolates were not detected with either target of the previously used *toxAB* assay (20), suggesting extensive genetic divergence of *tox*. NTTB isolates with known collection dates were obtained in 1971 to 2018, indicating sporadic and continued occurrence of NTTB *C. diphtheriae* and *C. ulcerans*. Overall, 5.9% (29/494) of nontoxigenic *C. diphtheriae* and *C. ulcerans* isolates were NTTB isolates.

Genomic analysis of NTTB isolates. Five unique putative mutations were observed in the *tox* coding region by whole-genome sequencing of NTTB isolates of *C. diphtheriae*, summarized in Table 3. Included among these were single nucleotide deletions within homopolymeric tracts at three different positions. The *tox* gene of a single *C. diphtheriae* biotype belfanti isolate was disrupted by insertion sequence element (ISE) IS*1132*. In four closely related *C. diphtheriae* biotype intermedius isolates, no putative mutations could be identified in *tox* or its promoter, including all intergenic positions upstream of the *tox* start codon, up to the preceding gene (350 bp in NCTC 13129).

Mutation identification in NTTB *C. ulcerans* isolates was limited by the lack of suitable reference sequences from toxigenic strains, most of which were phylogenetically disparate from the isolates here. For example, PC0090 and PC0640 each exhibited >12,900 single nucleotide polymorphisms (SNPs) relative to nontoxigenic strain FRC11 and >36,300 SNPs to toxigenic strain 131001. The *tox* gene and promoter sequences from PC0090 and PC0640 were identical to those in a draft assembly of 03-8664, an isolate recovered in France following zoonotic transmission (34). Similarly, NTTB isolate

TABLE 4 RT-PCR results for clinical specimens tested with current *Corynebacterium* triplex and previous *toxAB* assays[a]

Specimen source and type	No. of specimens	Current assay result			Previous assay result[b]		Culture result
		tox	Diph_*rpoB*	CUP_*rpoB*	*toxA*	*toxB*	
Oropharyngeal swab	56	−	−	−	−	−	−
	16	+	+	−	+	+	−
	14	+	+	−	+	+	+
	1	+	−	−	+	+	−
	2	+	+	−	−	−	−
	1	+	−	−	−	−	−
	1	+	+	−	−	+	−
Nasopharyngeal swab	10	−	−	−	−	−	−
Throat tissue	1	−	−	−	−	−	−
Pseudomembrane	1	−	−	−	−	−	−
Biopsy specimen	1	−	−	−	−	−	−
Bronchoalveolar lavage fluid	1	−	−	−	−	−	−
Total	105	35	33	0	31	32	14

[a]Culture was also performed, and all 14 isolates obtained from specimens were toxigenic. Diph_*rpoB*, *C. diphtheriae rpoB*; CUP, *C. ulcerans/C. pseudotuberculosis*.
[b]From reference 20.

PC0190 and toxigenic isolate PC0108 each differed from reference strain 210931 by >330 SNPs and from each other by 206 SNPs, including 126 nonsynonymous mutations, none of which appeared in *tox* or its promoter (Data Set S2). *C. ulcerans* NTTB isolate PC0365 and toxigenic isolate PC0364 were cultured from the pseudomembrane and throat swab, respectively, from the same patient. The two isolates differ by nonsynonymous mutation of three genes, but not *tox* (Data Set S2). Similarly, no amino acid changes were observed in *dtxR*, which encodes the diphtheria toxin repressor, in the unexplained NTTB *C. ulcerans* and *C. diphtheriae* biotype intermedius isolates.

Clinical specimens. The RNase P gene was detected in all specimens, confirming the presence of human DNA in the extracts. Thirty-five of 105 throat swabs were *tox* positive (Table 4), 33 of which were also positive for Diph_*rpoB*, all from international sources. Fourteen toxigenic *C. diphtheriae* biotype mitis cultures were isolated from RT-PCR-positive specimens (40%), demonstrating complete agreement of RT-PCR with culture-positive results. Three *tox*-positive specimens were not detected with the previous *toxAB* assay (20), and a fourth was only detected previously with the *toxB* target.

Clinical specimens were also tested with 4 μl template per reaction (water was reduced to 1.5 μl per reaction). This led to detection of two additional *tox*-positive specimens ($n = 37$). Also, C_T values for *tox* and Diph_*rpoB* were slightly lower in 85% of positive specimens when the use of 4 μl template was compared to the use of 2 μl template (data not shown), indicating a slightly increased sensitivity when the template amount is increased.

DISCUSSION

The CDC *Corynebacterium* triplex RT-PCR assay is an accurate and rapid tool for diphtheria diagnostics to identify clinically relevant species, screen isolates for confirmatory Elek testing, and provide fast reporting of isolates lacking *tox*. Testing demonstrated sensitive and specific detection of *tox*, and identification of *C. diphtheriae* and *C. ulcerans*/*C. pseudotuberculosis* within isolates and clinical specimens. The *tox* target described here, which bridges coding regions for toxin subunits A and B, is a more sensitive target than found in the previous *toxAB* assay (20), evidenced by improved detection of toxigenic *C. ulcerans* isolates and detection of *tox* in four specimens from suspected diphtheria patients that were negative with the previous assay. The RT-PCR assay also complements culture diagnostics by providing rapid presumptive diagnostics for clinical specimens, especially when responding to outbreaks in settings with limited culture availability.

A similar RT-PCR assay was described recently for use on the Rotor-Gene (Qiagen) or LightCycler 480 II (Roche) (24, 25). The same genes were targeted, and while *tox* and *C. diphtheriae rpoB* targets occur at different positions for the two assays, the *C. ulcerans*/*C. pseudotuberculosis rpoB* target positions overlap. In the updated protocol described by Badell et al. (25), an internal PCR control targeting the 16S rRNA gene replaces the previous green fluorescent protein target (24). Targeting a "universal" region within 16S rRNA gene enables confirmation of template DNA in isolates and specimens, reducing the possibility of false negatives due to poor DNA quality or PCR inhibition. However, the authors found that the 16S rRNA gene was detected in no-template controls (NTCs), possibly indicating the presence of bacterial DNA in the RT-PCR reagents (35). In contrast, no signal was detected in NTCs by using the RNase P gene in the current RT-PCR assay, performed in parallel to detect human DNA in clinical specimens as an external process control.

A clinical validation of the *Corynebacterium* triplex RT-PCR assay was not attempted at this time because of limited availability of specimens from suspected diphtheria cases in the United States. Analysis of 105 specimens indicated that RT-PCR provided more sensitive detection of diphtheria than culture diagnostics (Table 4), with no negative RT-PCR results in culture-positive specimens. Relatively low recovery of *C. diphtheriae* strains from suspected diphtheria cases (14/105, 13%) compared to RT-PCR (35/105 *tox* positive; 33%) is not unexpected. Possible contributing factors could be antibiotic use before specimen collection and time taken to transport specimens to the laboratory after collection. All positive specimens were obtained from international sources, which required additional transport time. Another limitation was noted for CUP_*rpoB* by the erroneous detection of two *C. renale* isolates obtained from nonhuman primates (data not shown). *C. renale* is not typically considered pathogenic to humans, and this rare cross-reaction is expected to have minimal impact on the efficacy of the assay (36). The Diph_*rpoB* target is highly specific to identify *C. diphtheriae*, the primary causative agent of diphtheria, as demonstrated in the results presented here (Table 2).

Molecular detection of *tox* is not indicative of diphtheria toxin production, as seen in the continued circulation of NTTB isolates of *C. diphtheriae* and *C. ulcerans*. The most commonly observed *tox* mutations in NTTB isolates examined here were deletions in homopolymeric regions, which are susceptible to strand slippage and thus potentially reversible (37). While homopolymer indels and ISE insertion likely disrupt encoded protein function (38), nonsynonymous SNPs remain difficult to "confirm" as determinants of NTTB, particularly given the nonsynonymous variation common among *tox* sequences from toxigenic references. The lack of detected mutations within *tox*, its promoter, or in the *dtxR* repressor in several isolates underlines the need for additional study of diphtheria toxin regulation. Little is known about the potential for NTTB isolates to regain toxigenicity, or even the frequency at which nontoxigenic isolates become lysogenized by *tox*-encoding bacteriophage. Recovery of closely related *C. ulcerans* NTTB and toxigenic isolates suggests that within-patient variability occurs during infection, further illustrating our limited understanding of this potentially dynamic phenotype. Such areas require further attention to improve diphtheria laboratory diagnostics, especially in the context of increased clinical identification of *C. diphtheriae* and *C. ulcerans* with MALDI-TOF mass spectrometry. Based on the extensive testing here, *tox* detection by RT-PCR provides an accurate indicator of toxigenic *C. diphtheriae* (or *C. ulcerans*/*C. pseudotuberculosis*) approximately 94% of the time. It is likely not feasible to design a single molecular test to confirm NTTB isolates based on the breadth of underlying mutations, both observed and as yet undetected. As a result, culture diagnostics with the Elek test remains the gold standard and only way to confirm the presence of toxigenic *Corynebacterium* species.

Conclusion. The CDC *Corynebacterium* triplex RT-PCR assay is an effective diphtheria diagnostic tool that provides rapid and sensitive probable-case determination. Culture diagnostics that include toxigenicity testing are still required for laboratory confirma-

tion of diphtheria because of the circulation of NTTB isolates and the potentially fluid state of *Corynebacterium* species toxigenicity.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.

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