Development, validation and implementation of a quadruplex real-time PCR assay for identification of potentially toxigenic corynebacteria

Aruni De Zoysa, Androulla Efstratiou, Ginder Mann, Timothy G. Harrison and Norman K. Fry

Respiratory and Vaccine Preventable Bacteria Reference Unit, Public Health England, Microbiology Reference Services, National Infection Service, London, UK

Toxigenic corynebacteria are uncommon in the UK; however, laboratory confirmation by the national reference laboratory can inform public health action according to national guidelines. Standard phenotypic tests for identification and toxin expression of isolates can take from ≥24 to ≥48 h from receipt. To decrease the time to result, a real-time PCR (qPCR) assay was developed for confirmation of both identification of Corynebacterium diphtheriae and Corynebacterium ulcerans/Corynebacterium pseudotuberculosis and detection of the diphtheria toxin gene. Target genes were the RNA polymerase β-subunit-encoding gene (rpoB) and A-subunit of the diphtheria toxin gene (tox). Green fluorescent protein DNA (gfp) was used as an internal process control. qPCR results were obtained within 3 to 4 h after receipt of isolate. The assay was validated according to published guidelines and demonstrated high diagnostic sensitivity (100 %), high specificity (98–100 %) and positive and negative predictive values of 91 to 100 % and 100 %, respectively, compared to both block-based PCR and the Elek test, together with a greatly reduced time from isolate receipt to reporting. Limitations of the qPCR assay were the inability to distinguish between C. ulcerans and C. pseudotuberculosis and that the presence of the toxin gene as demonstrated by qPCR may not always predict toxin expression. Thus, confirmation of expression of diphtheria toxin is always sought using the phenotypic Elek test. The new qPCR assay was formally introduced as the front-line test for putative toxigenic corynebacteria to inform public health action in England and Wales on 1 April 2014.

INTRODUCTION

Diphtheria is an acute human disease classically affecting not only the upper respiratory tract but also occasionally the skin (cutaneous diphtheria). It is caused by the action of diphtheria toxin (DT). There are currently more than 122 species of corynebacteria cited in the ‘List of Prokaryotic Names with Standing in Nomenclature’ (http://www.bacterio.net/index.html), but the only three species which are potentially toxigenic (i.e. capable of secreting DT) are Corynebacterium diphtheriae, Corynebacterium ulcerans and rarely Corynebacterium pseudotuberculosis. Diphtheria remains a serious health problem within many regions of the world with low vaccine coverage (Eastern Europe, Southeast Asia, South America and Africa) (Zasada, 2015). Immunization is one of the most successful and cost-effective health interventions known, and the World Health Organization (WHO) describes receipt of a third dose of diphtheria tetanus pertussis vaccine by age 12 months as a key indicator of immunization programme success (WHO, 2013). Diphtheria is uncommon in the UK and is rare in developed countries with good (diphtheria toxoid containing) vaccine coverage.

Between 1986 and 2013, 134 toxigenic C. diphtheriae and C. ulcerans (68 and 66 isolates, respectively) were submitted to the Diphtheria National Reference Laboratory (NRL) from England and Wales, and among these, 35 were from cases of ‘classical’ diphtheria (Public Health England, 2014). C. diphtheriae is the main aetiological agent of diphtheria. Patients presenting with the classical respiratory diphtheria have a swollen ‘bull neck’ and an adherent ‘pseudomembrane’...
in the respiratory tract mucosa. Previously considered a strictly human pathogen, *C. diphtheriae* can also infect animals, including cats, cows and horses (Corboz et al., 1996; Hall et al., 2010; Leggett et al., 2010).

*C. ulcerans* infections in humans can also be respiratory or cutaneous and usually involve close animal contact. Reservoirs include animals from farms and domestic and natural settings (Booth et al., 2015).

*C. pseudotuberculosis* is a known veterinary pathogen and infects ungulates, predominantly sheep and goats, although it may also infect horses, cattle and deer (Lipsky et al., 1982; Torres et al., 2013; Abebe & Sisay Tessema, 2015). Human infections with *C. pseudotuberculosis* are rare but have been reported particularly among those with close contact to animals due to occupation, e.g. sheep farmers in Australia (Torres et al., 2013; Heggelund et al., 2015), or vacation in a rural area (Join-Lambert et al., 2006).

Detection of toxigenicity is the most important test for the microbiological confirmation of a clinical diagnosis of diphtheria and also precipitates further public health action in England and Wales according to national guidance (Public Health England, 2015).

Identification of putative toxigenic corynebacteria by hospital microbiology laboratories in the UK is usually performed following primary culture on tellurite and blood agar (or selective tellurite-containing media). Colonies yielding a positive result in the catalase test, which are Gram-positive coryneform rods, may be further identified by conventional biochemical testing using commercial systems, e.g. API Coryne, VITEK microbial identification system (bioMérieux) or matrix-assisted laser desorption/ionization time of flight MS (Rennie et al., 2008; Neal & Efstratiou, 2009; Konrad et al., 2010; Farfour et al., 2012).

These methods can have good specificity; however, identification of *C. pseudotuberculosis* and differentiation from other corynebacteria, especially *C. ulcerans*, by standard biochemical methods are difficult (Heggelund et al., 2015). Toxin testing of putative isolates belonging to all three potentially toxigenic species is recommended, and in the UK, determination of toxigenicity requires submission to the NRL (Public Health England, 2015).

The Diphtheria NRL, London, has provided confirmation of identification of toxigenic corynebacteria for more than 29 years (Bonnet & Begg, 1999). Traditionally, characterization of pathogenic corynebacteria has been undertaken by the NRL using phenotypic methods, and demonstration of toxin production by the Elek test can take from ≥24 to ≥48 h from receipt of submitted isolate (Efstratiou & Maple, 1994).

A block-based PCR assay targeting the *tox* gene designed to screen for toxigenic *C. diphtheriae* was described by Pallen (1991) and subsequently evaluated on larger sets of clinical isolates of corynebacteria (Pallen et al., 1994). Since then, this assay has been used for specific studies on an ad hoc basis for referred isolates.

Real-time PCR (qPCR) assays using hydrolysis probes (e.g. TaqMan, Applied Biosystems) or dual-hybridization probes (e.g. LightCycler, Roche) targeting regions within the *tox* gene of *C. diphtheriae* and *C. ulcerans* have been described previously (Hauser et al., 1993; Mothershed et al., 2002; Schuhegger et al., 2008; Sing et al., 2011; Mancini et al., 2012). However, these are not multiplex assays, do not confirm species identity in a single reaction and do not contain an internal process control (IPC). Torres et al. (2013) described a multiplex (five-plex) PCR assay to identify and determine the potential toxigenicity of *Corynebacterium* spp. with zoonotic potential, but this was using the standard block-based (non-real-time) PCR approach. The advantages of a real-time multiplex PCR assay include data collection in the exponential phase of the reaction, a reporter fluorescent signal directly proportional to the number of amplicons generated, increased dynamic range of detection and no requirement for post-PCR processing.

We report here the development of a qPCR quadruplex Rotor-Gene Q (Qiagen) assay which simultaneously detects *tox* gene bearing and non-*tox* gene bearing *C. diphtheriae* and *C. ulcerans*. *C. pseudotuberculosis* in genomic DNA extracts from strains. The qPCR targets the RNA polymerase β-subunit-encoding gene (*rpoB*) and the *tox* gene. Detection of green fluorescent protein DNA (gfp) was used as an IPC to check for the presence of PCR inhibitors as previously described by Mentasti et al. (2015). The qPCR assay was validated according to local and other published guidelines for tests dependent on nucleic acid amplification and detection (Burk, 2010; Saunders et al., 2013).

Non-toxigenic *C. diphtheriae/C. ulcerans* usually lacks the entire DT gene, but rarely, some non-toxigenic strains may carry components of the *tox* gene. These strains, designated as non-toxigenic toxin gene-bearing corynebacteria (NTTB), can appear genotypically *tox* positive (as detected by PCR assays targeting the *tox* gene) but do not express the DT protein (detected using the Elek test), i.e. NTTB are tox*+* PCR positive but Elek negative (Mel’nikov et al., 2004; Zakikhany et al., 2014). A number of these strains (*n=4*) were also included in this evaluation.

The objectives of this study were to (i) design and develop a qPCR assay capable of detecting all three potentially toxigenic *Corynebacterium* spp. and detect the presence of the DT gene, (ii) validate the assay and (iii) implement into the NRL routine reference testing and on-call service following successful validation.

**METHODS**

**Bacterial strains.** Bacterial strains were obtained from the following sources: Public Health England (PHE), Colindale, London, UK; PHE National Collection of Type Cultures (NCTC), Salisbury, UK; Barts and the Royal London Hospital, London, UK; the CIP, Paris, France; and the American Type Culture Collection, Manassas, VA, USA. The toxigenic *C. diphtheriae* strain NCTC 10648 and non-toxigenic *C. ulcerans* NCTC 12077 were used as control strains to construct standard
curves and to establish efficiency, reproducibility and analytical sensitivity of this qPCR.

Panels were assembled to test specificity and consisted of (i) 84 non-target strains (12 genera, 47 species) (see Table S1, available in the online Supplementary Material) and (ii) 74 strains of corynebacteria, 40 toxigentic (C. diphtheriae, n=34; C. ulcerans, n=5; and C. pseudotuberculosis, n=1), 30 non-toxigentic (C. diphtheriae, n=29, and C. ulcerans, n=1) and 4 C. diphtheriae (3 from Lithuania and 1 from England) previously identified as conventional tox PCR positive (Pallen et al., 1994) but Elek negative, i.e. NTTB (see Table S2).

The identification of all strains was previously confirmed by standard methods and obtained from the culture collections described above. The identification and toxigenicity status of the corynebacteria were performed using phenotypic characteristics on Columbia blood, Hoyle’s tellurite and Tinsdale agar plates (PHE Media Services, Colindale); the API Coryne (bioMérieux), the modified Elek test (Engler et al., 1997), additional biochemical tests (e.g. nitrate reduction, glycogen hydrolysis) and DNA sequencing by rpoB gene (Khamis et al., 2004) for differentiation between C. diphtheriae biotypes and C. ulcerans/C. pseudotuberculosis were performed as required (Efstatiou & Maple, 1994). The presence/absence of the toxin gene in the potentially toxigeneric corynebacteria was also confirmed by the conventional tox PCR (Pallen et al., 1994).

**Genomic DNA extraction.** Extraction of DNA was performed using (i) the Wizard genomics DNA purification kit (Promega), (ii) a modified guanidinium thiocyanate method described previously (Pitcher et al., 1989; De Zoysa et al., 1995) or (iii) a boiling method as follows: a 1 µl loopful of bacterial growth was emulsified in 500 µl nucleic acid lysis buffer (Sigma) in a 1.5 ml sterile screw cap microfuge tube with O-ring. Genomic DNA was released by placing in a pre-heated heat block at 100 °C for 15 min. A similar tube containing 500 µl of nuclease-free water was included as a no template control (NTC) in each run. The microfuge tubes were centrifuged briefly for 1 min at 11 000 g. Five microlitre supernatant was used as template DNA in the qPCR assay. Control DNAs were quantified using the Qubit dsDNA broad-range assay kit with a Qubit fluorometer (Thermo Fisher Scientific).

**Primers and probes.** Oligonucleotide primers and probe were designed using the software Primer 3 (http://primer3.sourceforge.net/) to target rpoB and the tox gene. *In silico* specificity was assessed using the nucleotide query algorithm BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990). The green fluorescent protein gene (*gfp*) was used as an IPC as previously described (Murphy et al., 2007; Mentasti et al., 2015). Oligonucleotide sequences of primers and probes used in this study are shown in Table 1.

**Table 1.** Oligonucleotides, target genes, amplicon sizes and qPCR detection channel used

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide name</th>
<th>Sequence (5’–3’)</th>
<th>Amplicon fragment size (bp)</th>
<th>Rotor-Gene channel</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>C. diphtheriae</strong></td>
<td>dip_rpoBF</td>
<td>CGT TCG CAA AGA TTA CGG AAC CA</td>
<td>97</td>
<td>Yellow</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>dip_rpoBR</td>
<td>CAC TCA GGC GTA CCA ATC AAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CdhIP</td>
<td>HEX$_6$-AGG TTC CGG GGC TTC TCG ATA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCC A-BHQ1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C. ulcerans</strong></td>
<td>ulc_rpoBF</td>
<td>TTC GCA TGG CTC ATT GGC AC</td>
<td>98</td>
<td>Green</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>ulc_rpoBR</td>
<td>TCC AGG ATG TCT TCC AGT CC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CulcHP</td>
<td>FAM-CCA GCA GGA GGA GCT GGG TGA A-BHQ$_1$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>tox</strong></td>
<td>toxAF</td>
<td>CTT TCT TTC GTA CGG GAC GTA</td>
<td>117</td>
<td>Orange</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>toxAR</td>
<td>CTA TAA AAC CTT TTC CAA TCA TCG TC</td>
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<tr>
<td></td>
<td>diptoxHP</td>
<td>ROX$_6$-AAG GTA TAC AAA AGC CAA AAT</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTG GTA CAC AAG G-BHQ2</td>
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<tr>
<td><strong>gfp</strong></td>
<td>gfp_FP</td>
<td>CCT GTC CTT TTA CCA GAC AAC CA</td>
<td>77</td>
<td>Red</td>
<td>Mentasti et al. (2015)</td>
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<tr>
<td></td>
<td>gfp_RP</td>
<td>GGT CTC TCT TTT CGT TGG GAT CT</td>
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<td></td>
<td></td>
<td>Cy5$_6$-TAC CTG TCC ACA CAA TCT GCC TCT TCG-BHQ2</td>
<td></td>
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</tr>
</tbody>
</table>

*RNA polymerase β-subunit-encoding gene.
†DT gene.
‡Green fluorescent protein gene.
§Hexachlorofluorescein.
¶Black-hole quencher.
||6-Carboxyl-X-rhodamine.
#Cyanine 5.
RESULTS

In silico specificity

BLASTn analysis (performed on 1 January 2013) of the oligonucleotides targeting *C. diphtheriae rpoB* and the DT gene (*tox*) gave maximum similarity (100%) over all nucleotides to the intended target only, with one exception. The *tox* probe (diptoxHP) had one mismatch with *C. diphtheriae* strain BH8 (GenBank CP003209).1. BLASTn analysis of the oligonucleotides targeting *C. ulcerans rpoB* gave maximum similarity to *C. ulcerans* and the *C. pseudotuberculosis rpoB* gene.

Analytical specificity

None of the 84 (12 genera, 47 species) non-target organisms showed any cross-reactivity in the qPCR for the targets *C. diphtheriae rpoB*, *C. ulcerans rpoB* and *tox* as assessed by lack of any fluorescent probe signal in the respective channels (Table S1). Successful amplification of the IPC was demonstrated in replicates from all 84 organisms tested in the red channel of the Rotor-Gene, demonstrating that the lack of signal described above was not due to PCR inhibition. As expected, amplification of the target genes *rpoB* and/or *tox* was not observed in the NTC samples, but amplification was observed in the IPC channel in all cases. Fig. S1 shows an example of the results obtained with the IPC. The results of analysis of the 40 toxigenic strains tested by qPCR showed 100% correlation with the results obtained with both phenotypic tests and the conventional PCR for the amplification of the *tox* gene (Table S2). The four NTTB *C. diphtheriae* isolates which were non-toxigenic by the phenotypic Elek test but positive in the conventional tox PCR were also positive for the toxin gene by the multiplex qPCR assay (Table S2).

All 50/50 (100%) routine submissions of putative toxigenic corynebacteria cultures processed in parallel from September 2013 to March 2014 by standard phenotypic methods and the new qPCR assay showed 100% agreement. Of the four isolates with qPCR results of *C. diphtheriae/C. ulcerans*/*C. pseudotuberculosis* not detected, three were cysteinase negative, and the colonial morphology was not consistent with corynebacteria, and one did not grow on the Tinsdale selective media.

Sensitivity

LOD was calculated empirically and by Probit analysis (Finney & Stevens, 1948) as ≤1 genome copy per reaction for the *C. diphtheriae rpoB*, *C. ulcerans rpoB* and the toxin gene (*tox*); see Figs S2 to S4.

PCR efficiency

The average percentage qPCR efficiency for *C. diphtheriae*, *C. ulcerans* and the *tox* gene is 90.9, 89.0 and 91.6%, respectively.

The results of the calculation of the metrics of the assay were as follows: diagnostic sensitivity, 100% [TP/(TP+FN); 42/(42+0)] compared to Elek (Tables S2 and S3) and 100% [40/(40+0)] compared to the block-based tox PCR (Table S2), i.e. no false-negative results (qPCR tox negative, Elek positive or qPCR tox negative, PCR tox positive) were reported. If the NTTB are excluded, then the diagnostic specificity compared to phenotypic identification is 100% [TN/(TN+FP); 84+30+48/(84+30+48)+0] (Tables S1–S3). If the NTTB are considered as 'FP', then the diagnostic specificity is 98% [162/(162+4)]. Similarly, compared to phenotypic identification with and without the NTTB, the qPCR diagnostic accuracy is 100% [(TP+TN)/(TP+FP+TN +FN); (42+162)/(42+0+162+0)] versus 98% [(42+162)/(42+4+162+0)]; the positive predictive value is 100% [TP/(TP+FP); (42/42+0)] versus 91% [42/(42+4)], and the negative predictive value remains 100% [TN/(TN+FN); (162/ (162+0)].

Following review of all the data and completion of local validation reports, the assay was deemed fit for purpose and formally launched on 1 April 2014. Stakeholders were notified of changes to the diphtheria reference laboratory service commensurate with the publication of new interim guidelines for the public health control and management of diphtheria in England and Wales, prior to publication of the final version (Public Health England, 2015).
DISCUSSION

We describe the design and validation of a quadruplex qPCR on the Rotor-Gene Q (Qiagen) for confirmation of the potentially toxigenic species C. diphtheriae, C. ulcerans and C. pseudotuberculosis and the detection of the DT gene tox. The gfp assay was successfully included as an IPC in this assay as previously described by Mentasti et al. (2015). No inhibition was observed, indicating that the boiling extraction method used was satisfactory and not refractory to amplification.

The efficiency and LOD of the assay were calculated for each of the three qPCR targets. Average percentage efficiencies of 90.9, 89.0 and 91.6% were demonstrated which were considered acceptable (Saunders et al., 2013). The LOD was ≤1 copy per reaction for all three targets (C. diphtheriae ropB, C. ulcerans ropB and the DT gene tox, which was also considered acceptable analytical sensitivity). With appropriate validation, the assay could also be applied directly to extracted clinical specimens.

The assay was demonstrated to be 100% specific for C. diphtheriae and C. ulcerans/C. pseudotuberculosis. The results of testing for the presence of the DT gene with the qPCR assay were 100% concordant with that of the conventional PCR of Pallen et al. (1994). However, demonstration of the presence of the toxin gene by PCR cannot be used to establish biological toxin expression. The four NTTB strains were qPCR tox gene positive but Elek negative. Thus, toxigenicity (i.e. toxin expression) must be established by the phenotypic Elek test. The results of parallel (phenotypic and qPCR) testing of the 50 strains received between September 2014 and March 2014 were 100% concordant.

The qPCR assay is rapid (3–4 h to result from receipt of isolate), specific and sensitive. The NTTB (i.e. PCR tox positive, Elek negative) strains are a known confounder, which is why following a PCR/qPCR tox-positive result, toxin expression must always be confirmed by the Elek test.

The major advantage of the qPCR presented here is the greatly reduced time to report a result, where public health action is warranted, or to exclude the presence of a toxin gene-bearing strain and so prevent unnecessary action. Although the possibility of false-negative qPCR for toxigenic C. ulcerans has been reported (i.e. qPCR tox negative, Elek positive) (Cassiday et al., 2008), no such result was observed using the assay described here. The most likely explanation is that the current assay targets a distinct region of the DT A gene which shows a much lower degree of sequence variation in the oligonucleotide binding sites compared to that of Nakao et al. (1996) and Mothershed et al. (2002).

The two main limitations of the assay presented here are the inability to determine expression of DT if (a portion of) the tox gene is present and the lack of a definitive identification of C. ulcerans and C. pseudotuberculosis. After review by the PHE Diphtheria Guidelines Working Group (comprising experts from PHE, Public Health Wales, the Animal Health and Veterinary Laboratories Agency, hospitals, WHO Global Collaborating Centre for Reference and Research on Diphtheria & Streptococcal Infections and academia), it was decided that a tox-positive qPCR result was sufficient to initiate public health action prior to obtaining a confirmatory result from the Elek test. The rationale was that although the true prevalence of NTTB is unknown (as their presence is only revealed by the combined use of PCR assays targeting the tox gene and the Elek test), the available data for England and Wales indicate that it is low. Since the assay was deployed on 1 April 2014 to 31 December 2015, five NTTB strains were isolated in England (between August and October 2014), three were epidemiologically linked and two were linked geographically (authors’ unpublished data). During this 21 month period, 135 strains were confirmed by the NRL as belonging to one of the three species, C. diphtheriae (n = 118), C. ulcerans (n = 16) and C. pseudotuberculosis (n = 1), of which 21 were both tox qPCR positive and Elek positive. Prior to this, four NTTB strains of C. diphtheriae were isolated from humans in the UK between March 2011 and June 2012 and also one from a companion animal (cat) in Belgium in June 2012 (Zakikhany et al., 2014). Although risk of reversion by NTTB to a fully toxigenic (expressive) state may also be considered low, current PHE guidance recommends treatment by antibiotics in the same way as fully toxigenic (i.e. Elek positive) strains (Public Health England, 2015).

In addition to describing a real-time assay for differentiation of tox-bearing C. diphtheriae and C. ulcerans on the LightCycler (Roche) platform (Sing et al., 2011), this group also developed and evaluated an assay on the TaqMan (ABI) platform that reliably detected both C. diphtheriae and C. ulcerans tox, overcoming an issue of a previously published real-time tox PCR that detected only C. diphtheriae tox while missing C. ulcerans tox (Schuhegger et al., 2008). This serves to illustrate the importance of constant review of molecular assays, particularly as new target sequence data become available.

The performance of the qPCR described here is continually monitored by PHE’s internal quality assurance system, external quality assessment programmes when available (Both et al., 2014) and audit. Since the introduction of the qPCR, we have now tested a total of 35 C. ulcerans strains (the identities of which were all also confirmed by phenotypic testing). All of these isolates gave the expected result in the qPCR (35/35). Clinical isolates of C. pseudotuberculosis are rare in the UK; however, we have now tested a total of five strains of C. pseudotuberculosis including three reference strains of animal origin NCTC 3450 (sheep), NCTC 4681 (sheep) and NCTC 4656 (horsee), all of which gave the expected result in the qPCR (5/5). Other isolates submitted for testing which were negative in the qPCR assay (i.e. gave a result of C. diphtheriae, C. ulcerans and C. pseudotuberculosis not detected) were identified as Corynebacterium amycolatum, Corynebacterium aurimucosum, Corynebacterium minutissimum and Exiguobacterium spp. by matrix-assisted laser desorption/ionization time of flight MS, and two further isolates were identified...
as Corynebacterium spp. (Corynebacterium propinquum/Corynebacterium pseudodiphtheriticum and Corynebacterium singularum/C. aurimucosum/C. minutissimum by 16S rRNA sequencing with 99% similarity).

Future work will investigate inclusion of a DNA extraction control, testing clinical specimens and evaluation of primers and probes specific for C. pseudotuberculosis in order to differentiate C. pseudotuberculosis strains from C. ulcerans strains using a modification of the current assay.

The qPCR assay described here provides the rapid establishment of the presence of the DT gene in submitted potentially toxigenic corynebacteria isolates, thus allowing timely public health action in the management of cases, clusters and contacts.

ACKNOWLEDGEMENTS

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