Rapid detection and molecular differentiation of toxigenic
Corynebacterium diphtheriae and Corynebacterium ulcerans strains by LightCycler PCR

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Abstract

The systemic symptoms of diphtheria are caused by the tox-encoded diphtheria toxin (DT) which is produced by toxigenic *Corynebacterium* spp. Besides the classical agent *C. diphtheriae*, the zoonotic pathogen *C. ulcerans* has increasingly been reported as an emerging pathogen for diphtheria. The reliable detection of toxigenic *Corynebacterium* spp. is substantial for both diphtheria surveillance in the public health sector and the clinical work-up of a patient with diphtheria-like symptoms. Since the respective *tox* genes of *C. diphtheriae* and *C. ulcerans* differ in both DNA and amino acid sequence from each other, both *tox* genes should be covered by novel real-time PCR methods. We describe the development and validation of a LightCycler PCR assay which reliably recognizes *tox* genes from both *C. diphtheriae* and *C. ulcerans* and differentiates the respective target genes by fluorescence resonance energy transfer (FRET) hybridization probe melting curve analysis.
**Introduction**

Diphtheria is caused by both toxigenic *Corynebacterium diphtheriae* and *C. ulcerans* strains harbouring lysogenic beta-corynephages bearing the *tox* gene. *tox* encodes for the diphtheria toxin (DT) which is responsible for the systemic symptoms of diphtheria. Since the beginning of modern bacteriology, *C. diphtheriae* is known as the etiologic agent of diphtheria, a disease already described in ancient times. In contrast, only recently *C. ulcerans* was recognized as an emerging zoonotic pathogen which has been found more often than *C. diphtheriae* in reported cases of both diphtheria and diphtheria-like disease in several Western industrialized countries with low diphtheria incidence (1, 11, 15, 21, 25). *C. pseudotuberculosis*, the causative agent of caseous lymphadenitis in sheep and goats, may also produce a DT-like toxin. Human infections to toxigenic *C. pseudotuberculosis* are extremely rare (25). Moreover, the complete *tox* gene of *C. pseudotuberculosis* has not been sequenced so far.

The reliable detection of toxigenic *Corynebacterium* spp. is substantial for both diphtheria surveillance in the public health sector and the clinical management of the individual patient presenting with diphtheria-related symptoms. While the traditional Elek test for detection of diphtheria-toxin is time-consuming or not available in many laboratories worldwide due to the lack of either expertise or antitoxin, real-time PCR offers a rapid tool to confirm the presence of the diphtheria-toxin encoding gene *tox* in an isolate or specimen. Both *tox* and DT from *C. diphtheriae* and *C. ulcerans* differ from each other in about 5% of their base pair and amino acid composition, respectively (22). Moreover, when compared to *C. diphtheriae tox* and DT (10) *C. ulcerans tox* and DT seem to be much more heterogenous, since the so far published 12 sequences of *C. ulcerans tox* and DT can be subdivided into 5 different *tox* and DT sequence groups, respectively (8, 17, 19, 20, 22).

The differences in *C. diphtheriae* and *C. ulcerans tox* allow a differentiation between these two related virulence genes by PCR (22). For the diagnostic work-up of a patient with diphtheria-like symptoms, however, it is important to have a fast, reliable and robust method.
detecting both tox genes with the same assay. While a conventional C. diphtheriae tox PCR first described in 1993 and used by many laboratories worldwide for identification of toxigenic corynebacteria amplifies both C. diphtheriae and C. ulcerans tox (5), real-time PCR assays allow a much faster laboratory diagnosis of tox-positive corynebacteria. Recently, we developed and evaluated a TaqMan-based real-time PCR assay reliably detecting both C. diphtheriae and C. ulcerans tox (18) thus overcoming the problem of a previously published TaqMan-based real-time tox PCR detecting only C. diphtheriae tox, but missing C. ulcerans tox (3, 9, 24).

The two most widely applied real-time PCR detection formats are TaqMan hydrolysis probes and LightCycler hybridization probes. Since diagnostic laboratories often have only one of these two real-time PCR systems available, we decided to develop and evaluate a LightCycler-based real-time PCR protocol allowing simultaneous C. diphtheriae and C. ulcerans tox genes detection as well as differentiation of the two tox genes from each other by fluorescence resonance energy transfer (FRET) hybridization probe melting curve analysis in a single assay.

Material and Methods

Control strains. Twenty tox-bearing strains of C. diphtheriae and C. ulcerans collected by the German Consiliary Laboratory on Diphtheria since 1999 (table 1) and two C. diphtheriae type strains (the toxigenic strain NCTC 10648 and the weakly toxigenic strain NCTC 3984) were included in this study. In addition, 70 DT-negative strains of various Corynebacterium spp., isolated from clinical and veterinary specimens (among them 14 C. diphtheriae subsp. gravis, 29 C. diphtheriae subsp. mitis, 11 C. diphtheriae subsp. belfanti and one C. ulcerans, respectively [18]) and the non-toxigenic type strain C. diphtheriae NCTC 10356 were used to determine the specificity of the real time PCR. Analytical specificity of the presented assay was further assessed by testing DNA preparations of 118 gram-positive and gram-negative
bacterial strains other than Corynebacterium spp. (13). All strains were characterised by
standard microbiological and biochemical procedures including API Coryne (bioMérieux,
Nürtingen, Germany) as described previously (20, 22). Moreover, 16S rRNA gene sequencing
was performed for corroborating the obtained species ID results. In addition, a 500 bp
fragment of the rpoB gene was sequenced and compared with the Genbank database (6). The
presence of the tox gene was investigated by a conventional tox-PCR described by Hauser et
al. (5). DT production was evaluated by a modified Elek Test as described previously (22,
26).

**Template DNA extraction.** Total DNA preparations of cultured bacterial organisms or
clinical specimens were prepared using the High Pure PCR Template Kit, Roche Diagnostics,
Mannheim, Germany. DNA concentrations were determined spectrophotometrically.

**Real-time PCR assay design.** Primer sequences were selected according to Sulakvelidze et
al., (23) in order to amplify a 248-bp fragment within the C. diphtheriae tox gene.
Additionally, the 1-nt mismatch (pos. 135 in the alignment; table 2) observed within the
annealing region of primer CD-toxF with recently deposited C. ulcerans tox gene sequences
(e.g. FJ 858272) was considered by applying a relatively low annealing temperature (50 °C)
in the corresponding real-time PCR thermocycle profile. To select LightCycler hybridization
probe sequences reliably covering the tox gene in both C. ulcerans and C. diphtheriae, all tox-
sequences currently available in the Genbank database - eight for C. ulcerans, seven for
human-derived C. diphtheriae and six for DT encoding prophages - were aligned. To allow
for a discrimination between the respective tox genes in C. ulcerans and human-derived C.
diphtheriae by subsequent LightCycler melting curve analysis, the 1-nt mismatch between
GenBank AY820132 (tox from C. diphtheriae) and FJ858272 (tox from C. ulcerans) was
considered when designing the corresponding sensor hybridization probe sequence.

**Oligonucleotides and PCR protocol.** Amplification primers CD-toxF (GAA AAC TTT
TCT TCG TAC CAC GGG ACT AA, pos. 118-146 in GenBank FJ858272), CD-toxR (ATC
hybridization probes CD-HP-3 (AAT A A TAC GAC GCT GCG GGA TAC-FL, pos.247-270 in GenBank FJ858272), and CD-HP-4 (LCreRed 640-CTG TAG ATA ATG AAA ACC CGC TC, pos.272-294 in GenBank FJ858272) were synthesised by Metabion (Munich, Germany) and TIB Molbiol (Berlin, Germany), respectively. Real-time PCR amplification mixtures contained Roche LightCycler Fast Start DNA Master HybProbe, 3 mM MgCl₂, primer and probes in a final concentration of 500 nM and 200 nM each, respectively, and 5 µl of template DNA. The LightCycler real time PCR thermoprofile consisted of a initial denaturation step at 95°C for 10 min, followed by a 50-cycle amplification profile: heating at 20°C/s to 95°C with a 10-s hold, cooling at 20°C/s to 50°C with a 20-s hold, and heating at 20°C/s to 72°C with a 30-s hold. Melting curve analysis (starting at 40°C) was performed with a temperature transition rate of 0.2°C/sec to determine the Tₘ values for the sequences targeted by the hybridization probes.

Within each run, 10³ copies of a recombinant plasmid (containing a modified segment of the expected amplicon) were tested in a separate reaction capillary as positive control. As negative control, PCR grade water was used instead of DNA template. To demonstrate the absence of PCR inhibitors, a 5 µl aliquot of the template DNA prepared from clinical samples was subjected to the LightCycler Control Kit DNA in a separate PCR reaction. Analytical sensitivity was determined at a 10-fold dilution series of C. diphtheriae NCTC 10648 DNA, diluted in pooled DNA preparations from five throat swabs of C. diphtheriae-negative patients (mimicking the natural background of the PCR assay when applied to clinical specimens) (2).

Results

Analytical specificity of the LightCycler PCR assay. All 20 tested tox-positive isolates (8 C. diphtheriae and 12 C. ulcerans) led to positive real-time PCR results, as evidenced by
probe fluorescence, whereas the 70 tox-negative Corynebacterium strains remained negative. Even after as few as 35 amplification cycles the generated LightCycler curves allowed the clear discrimination between tox-positive and –negative Corynebacterium strains. The specificity of this primer and probe combination was further evaluated with various gram-positive and -negative organisms other than C. diphtheriae or C. ulcerans (n = 118). Since all of these isolates tested negative, analytical specificity was determined as 100 %.

**Analytical sensitivity.** Systematic testing of DNA dilution series revealed a LOD (lower limit of detection) of 100 fg template DNA per 20-µl PCR reaction at three out of three independent replicates (2). A template input of 100 fg of bacterial chromosomal DNA equals to 30 to 40 genome copies (calculation was based on the known genome size of C. diphtheriae). Inhibition events were not observed with any of the investigated DNA preparations (pseudo throat swabs) mimicking the natural background of the PCR assay. In comparison to the conventional tox-PCR (5), the presented LightCycler PCR assay turned out to be about 25 times more sensitive.

**Melting curve analysis.** LightCycler melting curve analysis revealed characteristic melting points for tox-positive isolates. A T_m of 62 °C was observed for all of the investigated tox-positive C. diphtheriae strains (n= 8), whereas a T_m of 60 °C was observed for all of the investigated tox-positive C. ulcerans strains (n= 12). Figure 2 shows a graphical plot of LightCycler melting curve analysis with a representative collection of tox-positive isolates.

**Sequencing of real-time PCR products.** The amplicons of the tox-specific real-time PCRs performed on both C. ulcerans and C. diphtheriae strains were subjected to DNA sequencing in order to confirm the results of melting curve analysis on sequence level. Obtained sequences were analyzed employing NCBI Blast search and "pileup" (from the HUSAR sequence analysis package at http://genius.embnet.dkfz-heidelberg.de/) to construct a multiple alignment with representative sequences for each of the investigated species. The species
identity and the observed nucleotide differences in the respective target sequences were confirmed and an alignment of selected tox gene sequences is shown in Figure 1.

Discussion

Application of current real-time PCR technology allows a much more rapid identification of specific target genes than conventional PCR. As a consequence, many pathogen- or pathogenicity factor- specific PCR protocols have recently been adopted or newly developed for real-time PCR platforms. Especially in the field of medical microbiology, the availability of fast and reliable diagnostic results has considerable impact on guiding therapy and prophylaxis against important infectious diseases. In the last few years several new sequences of tox genes from C. ulcerans were published in the GenBank database which differed substantially from those of C. diphtheriae tox (19, 22). When trying to amplify tox genes by previously published conventional and TaqMan-based real-time PCR assays, Cassiday et al. found that the conventional tox PCR detects both C. diphtheriae and C. ulcerans tox genes, while their TaqMan-based real-time PCR assay proved to be insufficient in detection of C. ulcerans tox due to mismatches in primer and probe binding regions (3). These limitations were overcome by a novel TaqMan-based PCR assay recently developed by our group (18).

Considering that many diagnostic laboratories are only running one of the two most widely used real-time PCR concepts, i.e. TaqMan and LightCycler PCR, reliable protocols for both detection systems are needed. We therefore developed and evaluated a LightCycler PCR assay which reliably detects all so far published human-derived C. diphtheriae and C. ulcerans tox genes. Our assay is able to detect the presence of either tox gene in clinical strains with a detection limit of less than 40 genome copies. All 20 DT gene harbouring Corynebacterium spp. strains collected at the German Consiliary Laboratory for Diphtheria tested clearly positive in our real-time tox PCR as well as in the conventional C. diphtheriae tox PCR described by Hauser et al. (5). Moreover, the previously designed C. ulcerans tox-
specific PCR (23) yielded positive results for the 12 toxigenic C. ulcerans strains analysed in this study, while it was negative in all 8 tox-bearing C. diphtheriae strains. In addition, the novel LightCycler PCR and the conventional tox PCR by Hauser et al. (5) were congruent in all tox-positive and tox-negative Corynebacterium strains tested.

Although the real-time PCR assay described in the present study was found to be a reliable and rapid method for detecting the presence of tox, both physicians and microbiologists have to be aware that an Elek test must be performed on tox-positive isolates to test for the production of a functional DT. Similarly to the long known tox-bearing, but non-toxinogenic C. diphtheriae strains, the existence of tox-positive, Elek test-negative C. ulcerans strains has been described by us (22) and recently confirmed by others (3).

Besides detection of tox from both C. diphtheriae and C. ulcerans, LightCycler melting curve analysis allowed clear differentiation between the C. ulcerans tox gene and at least all so far published human-derived C. diphtheriae tox genes without any extra cost or physical manipulations.

Recently, Hall et al. identified a novel group of C. diphtheriae strains isolated from a cluster of four domestic cats within a single household in the USA (4). As these feline isolates harbour a variant C. diphtheriae tox sequence (which has higher sequence identity to the C. ulcerans tox sequence than to tox sequences of human-derived C. diphtheriae strains) and the sequence similarity of the species marker gene rpoB is less than 98 % compared to C. diphtheriae type strains, these feline isolates might represent a new subspecies of C. diphtheriae. Due to a 1-nt deletion at position 55 of the tox gene (according to GenBank FJ376656, FJ422272, FJ422273 and FJ422274) and a cytosine-to-thymine substitution at position 74, which would prematurely terminate the resulting peptide at aa position 25, these novel feline C. diphtheriae strains are not able to functionally express tox thus being nontoxinogenic (4). Therefore, the clinical significance of these strains in humans is not yet known; due to the lacking diphtheria toxin production it might be presumed that these strains...
will not pose a real diagnostic problem in the work-up of a patient presenting with diphtheria-like symptoms. However, it should be mentioned that its residual gene sequence (which is almost identical to the *C. ulcerans* *tox* sequence within the amplified segment between positions 118 to 366 of the respective GenBank entries [see FJ376656 in Figure 1]) will presumably be covered by the primers of the PCR assay presented in this study- and the resulting amplicons will consequently be misclassified as *C. ulcerans* *tox* gene by LightCycler melting curve analysis.

Based on the results of our study, the novel LightCycler real-time PCR assay is reliably detecting the *tox* genes of *C. diphtheriae* and *C. ulcerans* strains. Being about 25 times more sensitive than a conventional *tox*-PCR is certainly a diagnostic benefit when applying the presented real-time PCR assay for direct testing of clinical samples. Moreover, it is able to differentiate between *C. ulcerans* and *C. diphtheriae* *tox* genes by performing a simple melting curve analysis subsequent to the real-time amplification and detection process. The potential cross-reaction which could be expected with the slightly variant *tox* gene of the recently described nontoxigenic *C. diphtheriae* strains is addressed. Assuming that the occurrence of these variant feline strains is still considered as a sporadic event and considering that all of the established PCR protocols targeting a segment within the *tox* gene should equally be affected, a potential misclassification should not interfere with the diagnostic specificity of the presented real-time PCR protocol in routine medical microbiology. In conclusion, our study as well as the work of other groups illustrates the need of critically re-evaluating the analytical specificity of established real-time PCR assays for the detection of given bacterial targets and adjusting them in the case when any limitations in assay specificity or sensitivity are getting apparent. Since human clinical isolates of toxigenic *C. pseudotuberculosis* are extremely rare and the sequence of its *tox* gene is not yet available, we were not able to include toxigenic *C. pseudotuberculosis* strains in our PCR study.
Acknowledgements

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References


Table 1: tox-positive Corynebacterium spp. strains and control strains tested in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Year</th>
<th>Source/ gender/ age</th>
<th>Elek test</th>
<th>tox PCR</th>
<th>LightCycler PCR assay</th>
<th>Reference</th>
</tr>
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<tbody>
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<td>1</td>
<td>C. diphtheriae mitis</td>
<td>1997</td>
<td>Human/m/3</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
<td>14</td>
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<tr>
<td>107</td>
<td>C. ulcerans</td>
<td>2007</td>
<td>Cat/ND/ND</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
<td>18</td>
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<td>109</td>
<td>C. ulcerans</td>
<td>2007</td>
<td>Human/w/ND</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
<td>18</td>
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<td>110</td>
<td>C. ulcerans</td>
<td>2007</td>
<td>Human/w/ND</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
<td>18</td>
</tr>
<tr>
<td>111</td>
<td>C. diphtheriae mitis</td>
<td>2007</td>
<td>Laboratory trial DIPNET 2007</td>
<td>negative</td>
<td>positive</td>
<td>positive</td>
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<tr>
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<td>C. diphtheriae gravis</td>
<td>2007</td>
<td>Laboratory trial DIPNET 2007</td>
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<td>positive</td>
<td>positive</td>
<td>12</td>
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<tr>
<td>114</td>
<td>C. ulcerans</td>
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<td>Laboratory trial DIPNET 2007</td>
<td>positive</td>
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<td>negative</td>
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<td>positive</td>
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<td>positive</td>
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<tr>
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<tr>
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<td>C. ulcerans</td>
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<td>173</td>
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<td>179</td>
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<td>Human/m/64</td>
<td>negative</td>
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<td>positive</td>
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<td>Human/w/61</td>
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<td>positive</td>
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<tr>
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</table>

1 The tox PCR was performed as described previously (14). 2 m, man; w, woman; ND, not determined; DIPNET, Diphtheria Surveillance Network.
Figure 1: Multiple sequence alignment of *C. diphtheriae* (GenBank AY820132) and *C. ulcerans* *tox* genes (GenBank FJ858272). Annealing regions of primer and LightCycler hybridization probe sequences of the novel PCR assay are indicated together with the consistent 1-nt mismatch at pos. 294 (GenBank FJ858272) discriminating between the *tox* genes of both species. GenBank FJ376656 was included as a representative *tox* gene sequence of the recently described feline *C. diphtheriae* isolates (4) that will be covered but misidentified as *C. ulcerans* by T<sub>m</sub>-analysis of the presented PCR assay.
Figure 2: LightCycler amplification curves of representative *tox*-positive *Corynebacterium* spp. strains. Results obtained with a representative collection of 9 *tox*-positive *C. ulcerans* strains and 6 *tox*-positive *C. diphtheriae* strains, including *C. diphtheriae* NCTC 10648, 2 *C. diphtheriae gravis* and 2 *C. diphtheriae mitis* strains, are depicted.
**Figure 3:** LightCycler melting curve analysis plot of representative tox-positive *Corynebacterium* spp. strains. Results obtained with a representative collection of 9 tox-positive *C. ulcerans* strains (T$_m$ $\sim$ 60 °C) and 6 tox-positive *C. diphtheriae* strains (T$_m$ $\sim$ 62 °C), including *C. diphtheriae* NCTC 10648, 2 *C. diphtheriae gravis* and 2 *C. diphtheriae mitis* strains, are depicted.