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Detection of Differences in the Nucleotide and Amino Acid Sequences of Diphtheria Toxin from *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* Causing Extrapharyngeal Infections

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While *Corynebacterium ulcerans* can mimic classical diphtheria, extrapharyngeal infections are extremely rare. Sequencing of the diphtheria toxin (DT)-encoding *tox* gene of two *C. ulcerans* isolates from extrapharyngeal infections revealed differences from *C. diphtheriae* DT sequences, mainly in the translocation and receptor-binding domains. *C. ulcerans* supernatants were much less potent than supernatant from *C. diphtheriae*. A *C. ulcerans* DT-specific PCR is described below.

Among the pathogenic nondiphtheria corynebacteria, *Corynebacterium ulcerans* has only rarely been reported to cause disease in humans (8, 15). Nearly all cases which had come to clinical attention were characterized by pharyngeal infections mimicking classical diphtheria. This has been explained by the fact that in similarity to *Corynebacterium diphtheriae* and *Corynebacterium pseudotuberculosis*, *C. ulcerans* may harbor lysogenic β-corynephages coding for the diphtheria toxin (DT) which is responsible for the systemic symptoms caused by *C. diphtheriae*. Recently, several reports of severe *C. ulcerans* infections causing pseudomembrane formation (3, 6, 14, 21) and the isolation of *C. ulcerans* from domestic cats (20) alerted public health professionals involved in diphtheria control in Europe and the United States and prompted a change in the guidelines on control of toxigenic *C. ulcerans* in the United Kingdom (J. M. White, N. S. Crowcroft, A. Efstratiou, K. Engler, G. Mann, and R. C. George, Abstr. Publ. Health Lab. Serv. Annu. Conf., abstr. 50, 2001). In contrast to *C. ulcerans* infections leading to classical diphtheria-like symptoms, extrapharyngeal manifestations of *C. ulcerans* are extremely rare (22).

As a consequence of increased awareness of potentially severe *C. ulcerans* infections, the German Consiliary Laboratory on Diphtheria, which was established at the Max von Pettenkofer-Institute in 1997, started to characterize and collect *C. ulcerans* strains sent from different German laboratories for further differentiation and DT determination. Since 1997, isolates from two cases of human *C. ulcerans* infections came to the attention of our institute. In both cases, patients presented with an extrapharyngeal manifestation of *C. ulcerans* infection. One patient died from a severe necrotizing sinusitis caused by a toxigenic *C. ulcerans* strain (designated A2911) detected by *tox* PCR and Elek testing (23). The other patient was infected by a *tox*-positive *C. ulcerans* strain named A6361.

Case reports. A 40-year-old homeless alcoholic and drug-using patient received a deep skin ulceration on his right leg after falling down an escalator in the station area of a major German city. The patient denied having had contact with animals (including cattle and sheep), visiting rural areas, and traveling outside Germany in the previous few months. There was no fever and only slight redness of the wound. A swab that was obtained from the skin wound showed coryneform, gram-positive rods after Gram staining. After 24 h of incubation on sheep blood agar at 37°C in 5% CO_2, whitish, only slightly hemolytical, shiny colonies grew as single microbial pathogens. Microscopical examination of these colonies revealed gram-positive, coryneform rods, partly arranged in palisades. They were catalase positive, showed a positive reaction for urease, were negative for pyrazinamidase, and fermented glucose, ribose, maltose, and glycogen (6). A search of the database of the API Coryne system (version 3.3.3) identified the bacterium as *C. ulcerans* (API code 0001326). Biochemical identification was confirmed by partial sequencing of a 524-bp fragment of the 16S rRNA gene (bases 8 to 532); the results revealed similarity of up to 99% between the sequence of this strain (designated A6361) and *C. ulcerans* reference sequences in the NCBI Blast database. Phospholipase D was present, as demonstrated by a positive reverse CAMP test (1, 23).

Since *C. ulcerans* can acquire lysogenic β-corynephages coding for the DT, we performed a PCR (using primers DT1 and DT2 for detection of *tox* [11]), which showed the presence of the DT gene in *C. ulcerans* A6361. Interestingly, *C. ulcerans* reference strain CCUG 2708 (identical isolates are NCTC 7910, DSM 46325, and ATCC 51799) (19) was *tox* negative by PCR. Moreover, a modified Elek test was carried out as described previously (23) for phenotypic confirmation of toxin production in *C. ulcerans* A6361; the test yielded a negative result on two different occasions. However, an immunochromatographic strip test developed for the detection of DT in bacterial cultures (detection limit, 0.5 ng/ml) (7) showed a

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TABLE 1. Amino acid sequence differences in DT of *C. diphtheriae*, *C. ulcerans* A2911, and *C. ulcerans* A6361

Amino acid position	Amino acid at indicated position		
	C. diphtheriae	*C. ulcerans* A2911[a]	*C. ulcerans* A6361[a]
2	Ser	Asn	Asn
67	Thr	Thr	**Ala**
116	Val	Ile	Ile
183	Ala	**Glu**	**Glu**
210	Ala	**Ser**	**Ser**
233	Val	Ala	Ala
277	Gln	**Arg**	Gln
294	Thr	**Val**	**Val**
296	Pro	**Ser**	**Ser**
305	Ala	**Ser**	**Ser**
314	Ile	Val	Val
317	Glu	**Lys**	**Lys**
378	Ile	Leu	Leu
415	Leu	Val	Val
417	Asp	**Gly**	**Gly**
421	Val	Ala	Ala
432	Arg	Lys	Lys
491	Gly	**Asp**	**Asp**
492	Asp	**Ala**	**Ala**
493	Val	**Thr**	**Thr**
500	Ser	Thr	Thr
518	Arg	Thr	Arg
527	Asn	**Asp**	**Asp**
529	Ile	**Thr**	**Thr**
530	Ser	**Pro**	**Pro**
531	Ser	Leu	Leu
532	Asp	**Ser**	**Ser**
535	Gly	**Asp**	**Asp**
556	Phe	Ala	Ala
558	Ile	Val	Val

[a] Boldface characters indicate differences in amino acid class.

slightly positive result for *C. ulcerans* A6361 and a clearly visible line was seen on the test strip for toxinogenic *C. diphtheriae* NCTC 10648. The line obtained by performing the test on *C. ulcerans* strain A2911 showed an intensity level between those for *C. ulcerans* A6361 and the *C. diphtheriae* reference strain. Antimicrobial susceptibility testing was done using the E test (AB Biodisk). The assay was performed on cation-adjusted Mueller-Hinton II blood agar (BBL) (supplemented with 5% [vol/vol] sheep blood) according to the manufacturer's instructions. In the absence of standardized breakpoints for *Corynebacteria*, sensitivity to antimicrobials was determined using the criteria for *Streptococcus* spp. other than *Streptococcus pneumoniae* (17). When this assessment method was used, *C. ulcerans* A6361 was found to be susceptible to penicillin, ampicillin, oxacillin, cefuroxime, erythromycin, clindamycin, gentamicin, ciprofloxacin, vancomycin, and linezolid.

After oral treatment with ciprofloxacin (500 mg per day for 10 days), the wound healed without sequelae. A history of a documented vaccination against diphtheria could not be obtained from the patient.

Since no sequence data on *tox* from *C. ulcerans* could be found in the literature and in the relevant databases (http://www.ncbi.nlm.nih.gov:80/BLAST and http://www.embl-heidelberg.de), we sequenced the complete *tox* gene (1,683 bp) from the two *C. ulcerans* strains and compared the obtained sequences with the *tox* sequence from *C. diphtheriae* (GenBank accession number V01536 and the identical sequences at accession numbers K01722 and X00703). The sequences of both *tox* genes from the two *C. ulcerans* strains differed from the published *C. diphtheriae tox* sequence in 77 base pairs, leading to 27 different amino acids (Table 1) between the DT from *C. diphtheriae* and those from both *C. ulcerans* strains. Therefore, DT from *C. ulcerans* and *C. diphtheriae* are about 95% identical on both the nucleotide and the amino acid levels. While only one and three differing amino acids are located in the signal and the catalytic (C) region of the A fragment of DT (4, 12), respectively, 23 differences in the amino acid sequences of DT from *C. diphtheriae* and both *C. ulcerans* strains were found in the B fragment: 7 differences were situated in the translocation (T) region and 16 were situated in the receptor-binding (R) domain. Moreover, differences between the nucleotide and amino acid sequences of the two *C. ulcerans* strains were also detected (Table 1). The sequences of *tox* and DT from A6361 differed in 10 nucleotides and 3 amino acids from those of A2911, respectively. Interestingly, in four and six cases of nucleotide differences between *tox* genes from A2911 and A6361, the base pairs from *tox* genes of A2911 and A6361, respectively, were identical to those in the corresponding region in the *C. diphtheriae tox* gene. One of the three amino acids differing in the DT sequences of the two *C. ulcerans* strains was shared by strain A2911 and *C. diphtheriae*, while two of them were identical in the DT sequences of A6361 and *C. diphtheriae*. The amino acid in the DT sequence of strain A6361 that differed from the amino acid sequence in the DT sequences of strain A2911 and *C. diphtheriae* was on position 67 in the C part of the A fragment, while the two differing amino acids in strain A2911 were situated in the T and R domains, respectively.

To compare the in vitro toxicity of DT from *C. diphtheriae* with that from *C. ulcerans*, we performed cytotoxicity assays in a protocol similar to that described elsewhere (5). Briefly, isolates from *C. diphtheriae* NCTC 10648 and *C. ulcerans* A2911 and A6361 were grown in Elek broth (5 ml) at 37°C for 48 h to identical densities. Bacterial cells were removed by microfiltration with a 0.2 μm-pore-size filter, and serial dilutions of the filtrate were put on HeLa cells grown in 24-well plates. The plates were incubated for 4 days at 37°C and controlled microscopically on a daily basis. While the cell supernatant from *C. diphtheriae* NCTC 10648 was able to kill all HeLa cells at a titer of as low as 1:12,800, the supernatants from both *C. ulcerans* strains were much less active. *C. ulcerans* A2911 supernatant was toxic to the HeLa cells at a titer of 1:160, whereas not even the undiluted supernatant from *C. ul-*

TABLE 2. Synopsis of test results for *C. diphtheriae* NCTC 10648, *C. ulcerans* A2911, and *C. ulcerans* A6361

Strain	Results by indicated test			
	tox PCR	Elek	ICS[a]	Cytotoxicity (titer)
C. diphtheriae NCTC 10648	Positive	Positive	+++	1:12,800
C. ulcerans A2911	Positive	Positive	++	1:160
C. ulcerans A6361	Positive	Negative	+	Negative

[a] ICS, immunochromatographic strip; +++, strong visible band; ++, medium visible band; +, weak visible band.

cerans A6361 was able to kill the cells (Table 2). The cell-killing effect of both the *C. diphtheriae* NCTC 10648 and the *C. ulcerans* A2911 supernatants was completely inhibited by the addition of diphtheria antitoxin (0.01 IU/ml).

In the next step, we designed two different reverse primers, 1467R (5′-CGG CAA AAG GTT GTA GCA TC-3′) and 1586R (5′-GTC TAT GGA GCT CAA CGG AG-3′), for specific detection of *C. ulcerans* DT but not of *C. diphtheriae* DT. PCR was performed for primers DT1 and DT2 as described previously (11). While DT PCR using primers DT1 and DT2 gave positive results for *C. diphtheriae* NCTC 10648 and both *C. ulcerans* strains, the primer combination DT1 and 1467R and the primer combination DT1 and 1586R yielded a positive result only for *C. ulcerans* strains A2911 and A6361.

C. ulcerans was first isolated in 1926 by Gilbert and Stewart from human throat lesions (9). In 1995, Riegel et al. proposed *C. ulcerans* as a distinct species within the *C. diphtheriae* group on the basis of molecular analysis of genomic DNA (19). *C. ulcerans* commonly causes bovine mastitis (8). Several cases of human infection have therefore been linked to the drinking of unpasteurized milk from cows or goats (2). However, as with our two patients, most of the recent cases reported in the literature have not been associated with exposure to cattle or raw milk (3, 13, 21, 22). The observation that *C. ulcerans* infections can mimic classical diphtheria has been linked to the ability of *C. ulcerans* to produce DT similarly to *C. diphtheriae* (and *C. pseudotuberculosis*). Since the nucleotide and amino acid sequence of DT from *C. ulcerans* is not known so far, we sequenced *tox* from *C. ulcerans* A2911 and A6361 and compared it to *tox* from *C. diphtheriae*, which seems to be highly conserved in several different isolates. In a study in which the *tox* alleles from 72 isolates from the recent diphtheria epidemic in Russia and Ukraine were sequenced, only one silent point mutation in the region of *tox* encoding the A domain of DT and three silent mutations in the B domain were detected, suggesting a high level of conservation of DT in different *C. diphtheriae* strains (16). However, the differences between *tox* sequences in the *C. diphtheriae* and two *C. ulcerans* strains analyzed in our study also resulted in remarkable differences for DT on the protein level. Most of these differences were located in the T and R domain of the B fragment of DT.

After extensively reviewing published amino acid mutations leading to loss of function in *C. diphtheriae*, we were able to find only one amino acid exchange leading to loss of activity in mutated *C. diphtheriae* strains which was also present in either of the *C. ulcerans* strains analyzed in this study. This difference was at position 390 in the processed DT, where leucine is located in DT from *C. diphtheriae* and valine is located in DT from *C. ulcerans*. A mutation to phenylalanine in *C. diphtheriae* CRM107 in this position, together with a second mutation from serine to phenylalanine in position 525, was found to impair binding of DT (10). Although isolates of both *C. ulcerans* A2911 and A6361 were associated with severe extrapharyngeal infection, in a cytotoxicity assay bacterial supernatants of both strains were either unable to kill HeLa cells or were only able to do so at a relatively high concentration.

It might be speculated from the comparison of *C. diphtheriae* and *C. ulcerans* DT sequences that differences in the pathogenicities of *C. diphtheriae* and *C. ulcerans* regarding pseudomembrane formation and causation of classical diphtheria might result from impaired translocation and/or binding to the DT receptor, the heparin-binding epidermal growth factor precursor (12). Another explanation might be that *C. ulcerans* produces a smaller amount of DT than *C. diphtheriae* (15). In analogy to nontoxinogenic *C. diphtheriae* strains, which can also cause severe infections and have been considered to be an emerging infectious disease threat (8, 18), it may be concluded that the pathogenicity of *C. ulcerans* does not necessarily depend on the production of DT. Interestingly, it was possible to design primers for DT PCR that clearly differentiated *C. diphtheriae* DT from *C. ulcerans* DT. The two tested *C. ulcerans* DT PCRs could serve as a rapid tool to analyze isolates of *C. ulcerans* causing classical diphtheria for the presence of the regions in DT which were found to be specific for *C. ulcerans* DT in the present study.

Nucleotide sequence accession numbers. The GenBank accession numbers assigned to the *C. ulcerans tox* gene sequences determined in this study are as follows: AY141013 and AY1411014.

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