MICROBIAL PATHOGENICITY

Prevalence of *cdtABC* genes encoding cytolethal distending toxin among *Haemophilus ducreyi* and *Actinobacillus actinomycetemcomitans* strains

H. J. AHMED, L. A. SVENSSON, L. D. COPE*, J. L. LATIMER*, E. J. HANSEN*, K. AHLMAN, J. BAYAT-TURK, D. KLAMER and T. LAGERGÅRD

Department of Medical Microbiology and Immunology, University of Göteborg S-413 46 Göteborg, Sweden and *Department of Microbiology, Southwestern Medical Center, University of Texas, Dallas, USA

The aim of this study was to investigate the presence of the three *cdtABC* genes responsible for production of cytolethal distending toxin (CDT) in *Haemophilus ducreyi* and *Actinobacillus actinomycetemcomitans* strains. Of 100 *H. ducreyi* strains from the culture collection of the University of Göteborg (CCUG), 27 strains with low or intermediate cytotoxic titre (<1 in 10^4) and 23 of the remaining isolates with a high cytotoxic titre (≥ 1 in 10^4) were selected. Twenty-nine strains of *H. ducreyi* were isolated recently from patients with chancroid and 50 *A. actinomycetemcomitans* strains from patients with periodontitis. The cytotoxic activity on HEp-2 cells and the presence of *cdtABC* genes were studied by cytotoxicity assay of bacterial sonicates and PCR with primers specific for individual *cdtA*, *B*, and *C* genes of *H. ducreyi* in bacterial DNA preparations, respectively. All strains that manifested a cytotoxic titre in sonicate ≥ 1 in 100 possessed all the three *cdt* genes. Eighteen of the 50 strains selected from the culture collection were negative and 32 positive for *cdt* genes. As all strains with a high cytotoxic titre gave positive PCR results, it can be assumed that the remaining 50 strains, which have high cytotoxic titre, would have been positive as well. Thus, it can be estimated that 82% of the culture collection strains had *cdtABC* genes. Similarly, 24 (83%) of 29 recent *H. ducreyi* isolates expressed the CDT activity and displayed all *cdtABC* genes. Forty-three (86%) of 50 strains of the closely related *A. actinomycetemcomitans*, expressing a cytotoxic activity ≥ 1 in 100, also possessed all three genes. Furthermore, the nucleotide sequence of the *cdtABC* genes was highly conserved among *H. ducreyi* strains from different geographic areas. These results indicate that the majority of pathogenic *H. ducreyi* and *A. actinomycetemcomitans* strains express a CDT activity encoded by all three *cdtABC*.

Introduction

Haemophilus ducreyi causes chancroid, a sexually transmitted disease characterised by soft, painful, slowly healing genital ulcers [1–4]. Renewed attention has been focused on *H. ducreyi* since genital ulceration has been associated with HIV transmission in developing countries [5, 6]. *H. ducreyi* produces a cytotoxin that belongs to the cytolethal distending toxin (CDT) family. The *H. ducreyi* cytolethal distending toxin (HdCDT) has been shown to inhibit proliferation, induce cell enlargement and cause death of a number

of human cells and cell lines, e.g., HEp-2, HeLa, HaCaT, T cells, B cells and human fibroblasts [7–13]. It has been shown to induce a cell cycle arrest not only in the G2/M phase, but also in the G1 phase [14, 15]. *cdt* genes have also been identified in strains of *Escherichia coli* [16–18], some *Shigella* [19], *Campylobacter* [20–22] and *Helicobacter* [23] species and in the oral pathogen *Actinobacillus actinomycetemcomitans* [24–26]. All these toxins are encoded by an operon consisting of three genes (*cdtABC*), the products of which have molecular masses of *c.* 25, 30 and 20 kDa, respectively [10, 14]. *H. ducreyi* *cdtABC* genes encode proteins that closely resemble those comprising the CDT of *A. actinomycetemcomitans*, which also belongs to the family Pasteurellaceae and is involved in the pathogenesis of human periodontal disease. The amino acid identity between the

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Corresponding author: Dr T. Lagergård (e-mail: teresa.lagergard@microbio.gu.se).

CDTs from these two species is 91%, 96% and 94% for the *cdt A*, *B* and *C*, respectively [24, 26]. The functions of the individual CDT proteins have not been clearly established, but all three genes are necessary for the expression of cytotoxicity [13]. Recently, the *cdtB* gene product was reported to have a DNAase activity [27–29], which correlates well with the effect of CDTs on cell cycle progression. To date, the exact contribution of CDTs to pathogenesis has not been determined, although a correlation has been reported between the level of CDT expressed by *S. dysenteriae* and the amount of diarrhoea produced in a suckling mouse model [17]. Recently, a direct pathological effect of CDT was shown in *Campylobacter jejuni*; strains carrying a mutation in *cdtB* displayed impaired invasiveness in immunodeficient mice [30]. On the other hand an isogenic *cdtC* mutant of *H. ducreyi* was shown to be as virulent as its parent strain in the temperature-dependent rabbit model of experimental chancroid [31] and a CDT mutant retained the capacity to cause pustules in human volunteers [32]. The majority of *H. ducreyi* strains (89%) have been reported to exert cytotoxic activity on HEP-2 cells [33]; however, the prevalence of the *cdtABC* genes in *H. ducreyi* and in *A. actinomycetemcomitans* and correlation with cytotoxicity have not been studied.

The aim of this study was to investigate the cytotoxicity and prevalence of the *cdtA*, *B* and *C* genes of *H. ducreyi* and *A. actinomycetemcomitans* isolates.

Materials and methods

Bacterial strains and culture conditions

Fifty of 100 *H. ducreyi* strains used in a previous study [33], isolated from different parts of the world during 1950–1991 and placed in the Culture Collection of the University of Göteborg, (CCUG) were re-examined. These strains were selected as follows: 27 of the 100 strains, which manifested a low or intermediate cytotoxic titre (<1 in 10^4), and 23 strains, which represented the remaining *H. ducreyi* isolates with a high cytotoxic titre (≥ 1 in 10^4). The reference strains of *H. ducreyi* used in the previous studies, i.e., 35000, CCUG 4438 (CIP 542), CCUG 4436 (CIP A76) and CCUG 7470 (CIP 76118), were included. A further 29 *H. ducreyi* strains that had recently been isolated from patients with chancroid were also investigated. Twenty-four strains were isolated during 1997 from patients with genital ulceration diseases (GUD) visiting the City Health STD Clinic in Durban, South Africa and kindly provided by Professor W. Sturm. Five strains were isolated from a similar group of patients visiting the Kiwanja Mpaka and Igawilo Health Centers in Mbeya, Tanzania in March 1999. The identity of all these clinical isolates had been confirmed by PCR. Fifty strains of *A. actinomycetemcomitans*, isolated recently from patients with periodontitis, were obtained from

Professor Gunnar Dahlén, Department of Oral Microbiology, Odontology Faculty, University of Göteborg.

H. ducreyi and *A. actinomycetemcomitans* were grown on chocolate agar plates (GLV) containing brain heart infusion (BHI) agar 5%, horse blood 1%, horse serum 1.5%, yeast autolysate 0.06%, IsoVitaleX 0.015% and vancomycin 3 µg/ml at 33°C (*H. ducreyi*) or 37°C (*A. actinomycetemcomitans*) for 48 h, as described previously [7, 10].

Sonic preparation and DNA extraction

Strains cultivated on solid medium were sonicated, as described for *H. ducreyi* [7], or suspended in sterile distilled water, vortex mixed for 10 s with glass beads, boiled for 10 min and centrifuged at 3000 *g* for 5 min. The supernate was removed and stored at –20°C until required.

Cytotoxic assay

The cytotoxic activity of sonicates was tested with 24-h, 20–30% confluent monolayers of HEP-2 cells, as described previously [7, 10]. The cytotoxic titre was expressed as the dilution that gave $\leq 50\%$ of cell growth as compared with control (phosphate-buffered saline, PBS).

PCR

The identities of all *H. ducreyi* strains were confirmed by a modified version of a previously described PCR assay [34]. The primers used were the *H. ducreyi* 16S rRNA-specific sequence RPI 5'-CCCCTTTGCAGGTTTGCCGCCCTC-3', positions 1207–1230 and the non-specific sequence U3 5'-GTGCCTGCAGCCGC GGTAAT-3', corresponding to base positions 515–534 of the highly conserved U3 region of *Escherichia coli* 16S rRNA, to amplify a 758-bp fragment. The primers used to amplify individual *cdtABC* genes from the DNA of *H. ducreyi* strains are summarised in Table 1. For *cdtA*, primers 7 and 13 were used to amplify a 694-bp DNA fragment; for *cdtB*, primers 12 and 8 were used to amplify a 863-bp DNA fragment; and for *cdtC*, primers 1 and 4 were used to amplify a 593-bp DNA fragment (Innovagen AB, Lund, Sweden). The identity of the PCR products has been confirmed by sequencing, cloning and recombinant expression (unpublished observations and [29]). PCR was performed with 5 µl of the extracted DNA in 50 µl reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 2.5 U *Taq* polymerase, 0.2 mM of each deoxyribonucleoside triphosphate (dNTP) (Promega, Madison, WI, USA) and 10 µM of each primer. The amplification cycle comprised 28 cycles denaturation at 94°C for 2 min, annealing at 65°C for 1 min and extension at 72°C for 2 min. The PCR products were visualised by gel electrophoresis on an agarose 1% gel (DNA grade, Labora, Sweden) followed by staining

Table 1. Primers used in the PCR for the *cdtA*, *B* and *C* genes.

Name	Oligonucleotide sequence	Position in respective <i>cdt</i> gene
<i>CdtA</i> -7	5'-GGG,GGG,CTA,GTG,GAT,GGA,TCT,AAG,GAG,AGA, TAT,AAT,G-3'	497–523
<i>CdtA</i> -13	5'-GGG,GGG,GCT,CTT,AAT,TAA,CCG,CTG,TTG,CTT, CTA,ATA,CAG-3'	1190–1163
<i>CdtB</i> -12	5'-GGG,GGG,ATT,CTA,AGG,AGT,TTA,TAT,GCA,ATG, GGT,AAA,G-3'	1196–1223
<i>CdtB</i> -8	5'-GGG,GGG,AGC,TCT,TAG,CGA,TCA,CGA,ACA,AAA, CTA,ACA,G-3'	2058–2032
<i>CdtC</i> -1	5'-GGG,GGG,AAT,TCT,AGT,TTT,GTT,CGT,GAT,CGC, TAA,GGA,G-3'	2034–2059
<i>CdtC</i> -4	5'-GGG,GGG,CTA,GTT,AGC,TAC,CCT,GAT,TTC,TTC, GCA,CCG-3'	2626–2603

Sequences are expressed from 5' to 3' and base positions are according to Cope *et al.* [10]. The bases in italics represent non-homologous leader sequences carrying the restriction sites used for cloning.

with ethidium bromide and illumination with ultraviolet light.

Southern blot

Chromosomal DNA purified from 14 *H. ducreyi* strains with no or low cytotoxic titre was digested to completion with *Ava*I. These digests were resolved by agarose gel electrophoresis, transferred to nitrocellulose, and probed in Southern blot analysis as described previously [35]. The *cdtA*-specific DNA probe was prepared by using the oligonucleotide primers P3 and P6 [10] to amplify a 495-bp fragment of the *cdtA* gene from *H. ducreyi* strain 35000 chromosomal DNA. The 460-bp *cdtB*-specific probe and the 518-bp *cdtC*-specific probe were similarly amplified with the oligonucleotide primers P10–P12 and P14–P18, respectively [10].

Nucleotide sequence analysis of *cdtABC* genes of *H. ducreyi*

PCR was used to amplify the *cdtABC* gene cluster from four strains of *H. ducreyi* that expressed CDT activity. These included strain CCUG 7470 (isolated in France from an Algerian patient), strain 181 (isolated in Nairobi, Kenya), strain WPB506 (isolated in West Palm Beach, CA, USA) and strain PU1 (isolated in Bangkok, Thailand). PCR was accomplished with purified chromosomal DNA and *Taq* DNA polymerase (Promega Corp., Madison, WI, USA) according to the manufacturer's recommendations. The oligonucleotide primers used to amplify the *cdtABC* gene cluster were P1 5'-CCTTGTAAGATTATTCACCGTC-3' and P18 5'-ACCC TGATTTCTTCGCAC-3' [10]. Nucleotide sequence analysis was performed with Big Dye Terminator chemistry and a model 373A automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Results

Prevalence of *cdtABC* genes and cytotoxic activity of *H. ducreyi* strains

The PCR employed for verification of *H. ducreyi* species with the RPI and U3 primers to amplify the 16S rRNA gene showed that all strains tested were *H. ducreyi*.

The PCR technique was used to detect the three individual *cdtA*, *cdtB* and *cdtC* genes in the 50 selected strains of *H. ducreyi*. All three genes were detected in 32 of these 50 selected *H. ducreyi* strains; in the remaining 18 strains, all three genes were missing. The PCR results are exemplified by Fig. 1. All strains with a cytotoxic titre <1 in 100 had no detectable *cdtABC* genes. As the remaining 50 *H. ducreyi* strains (published previously) manifested a high (>1 in 10⁴) cytotoxic titre in the sonicate, it can be estimated that 82 of 100 strains of *H. ducreyi* in the culture collections express CDT activity. The results are summarised in Table 2.

To confirm whether or not *H. ducreyi* strains that either expressed no CDT activity or had very low toxin titres (<1 in 100) possessed the *cdtABC* gene cluster, a Southern blot analysis was performed with *cdtA*-, *cdtB*- and *cdtC*-specific probes against 10 *H. ducreyi* strains with very low cytotoxic titres. Four strains (35000, 7470, 87067, 29822) that readily expressed CDT activity were included as positive controls. The 10 strains with very low toxic activity did not bind *cdtA*, *cdtB* or *cdtC* probes, whereas the four control strains bound all three *cdt*-specific probes (not shown).

Among the 29 *H. ducreyi* strains isolated recently from

Fig. 1. Detection of individual *cdtA*, *cdtB* and *cdtC* genes of three *H. ducreyi* strains by PCR. Lanes 1, 5 and 9, DNA standards 1500 kb; 2–4, screening for *cdtA*; 6–8, screening for *cdtB*; 10–12, screening for *cdtC*. Lanes 2, 6 and 10, strain CCUG 7470; 3, 7 and 11, a patient isolate; 4, 8 and 12, strain CCUG 4438.

Table 2. Presence of *cdtABC* and cytotoxic activity of 100 *H. ducreyi* strains from culture collections and 50 *A. actinomycetemcomitans* strains from patients with periodontitis

Presence of gene cluster	Number of strains (n = 50) with cytotoxic titre	
	<100	≥100
<i>H. ducreyi</i>		
<i>Cdt ABC</i> (-)	18	0
<i>Cdt ABC</i> (+)	0	32
<i>A. actinomycetemcomitans</i>		
<i>Cdt ABC</i> (-)	7	0
<i>Cdt ABC</i> (+)	0	43

patients with genital ulcers, five did not have detectable *cdtA*, *B*, *C* genes and the sonicates from these strains were not toxic to HEp-2 cells (titre <1 in 100). Of the remaining strains, 24 possessed all three *cdt* genes and had cytotoxic activity ≥1 in 100. The percentage of fresh *H. ducreyi* isolates possessing all three *cdtABC* genes can be calculated to be c. 83%, which is similar in frequency to the sample of *H. ducreyi* strains obtained from the culture collections (82%). The similar prevalence of *cdtABC* genes in strains exposed to several culture passages and the fresh isolates indicates that the strains lacking these genes also occur naturally.

Prevalence of *cdtABC* genes and toxic activity in *A. actinomycetemcomitans* strains

The PCR method, with the same primers as for *H. ducreyi*, was employed to detect *cdtA*, *cdtB* and *cdtC* genes in 50 *A. actinomycetemcomitans* strains. All 50 sonicates were tested for cytotoxic activity on HEp-2 cells. Forty-three (86%) of 50 strains possessed all the three *cdtABC* genes and manifested cytotoxic activity of >1 in 100 (Table 2) indicating that the percentage of *cdtABC*-positive *A. actinomycetemcomitans* strains from patients with periodontitis was similar to that of *H. ducreyi* strains.

Comparison of *cdtABC* genes from various *H. ducreyi* strains

The nucleotide sequences of the *cdtABC* gene cluster from four *H. ducreyi* strains (7470, 181, WPB 506 and PU1) isolated in different geographic locations were determined. The nucleotide sequences of the *cdtA* and *cdtB* ORFs from all four strains were identical and were also identical to those of four other *H. ducreyi* strains described previously [10]. Among the *cdtC* ORFs, a single nucleotide change was found in one of the four strains analysed in this study. At position 2195 (based on the nucleotide sequence of the *H. ducreyi* strain 35000 *cdtABC* genes deposited in GenBank as accession no. U53215), the *cdtC* ORF of strain 7470 had a G in place of an A. This single nucleotide change resulted in the conservative substitution of

valine for isoleucine at position 161 in the amino acid sequence of the *cdtC* protein.

Discussion

The production of a CDT has been reported as a potential virulence factor of some gram-negative organisms including *H. ducreyi*. These CDTs inhibit cell proliferation and cause cell death in many cultured human cells, including normal human keratinocytes, fibroblasts and immune cells [12, 13]. However, the involvement of the toxin in the pathogenesis of chancroid has not yet been shown. Both in the temperature-dependent rabbit model and in the human model for chancroid, *H. ducreyi* strains with a mutation in the *CDT* genes were shown to be as virulent as their isogenic parent strains [34, 35]. However, these models do have limitations; animal cells are known to be not sensitive or less sensitive to HdCDT than human cells and, therefore, the pathogenesis in these rabbits could be different from that in man. In the human experimental model of *H. ducreyi* infection, only the primary stages of chancroid can be studied, i.e., the development of a pustule.

Eighty-nine of 100 *H. ducreyi* strains from different geographic areas were reported to have cytotoxic activity according to the criteria used in that study [33]. The present study demonstrates the presence of *cdtABC* genes in 82% of *H. ducreyi* strains, which correlates with cytotoxic activity. The presence of low cytotoxic activity in some previously reported strains [33], in which the *cdtABC* genes are absent, was probably due to non-specific action of other bacterial components in sonicates on HEp-2 cells.

In the present study, among the 12 ORFs from four *H. ducreyi* strains, there was only a single nucleotide difference. This same single nucleotide change was found in the *cdtC* ORF of strain RO18, one of the four additional *H. ducreyi* strains characterised previously [10]. Therefore, among the *cdtABC* ORFs from eight *H. ducreyi* strains, there was only a single nucleotide change and this occurred in just two strains. The conservation of the nucleotide sequence of the *cdtABC* genes among *H. ducreyi* strains is very striking. These data reinforce the conservation of this gene cluster among strains of this pathogen.

The origin of this highly conserved *cdtABC* gene cluster in *H. ducreyi* is unknown, but it must be noted that genes or incomplete ORFs that encode predicted proteins likely to function in transposition or recombination were detected within 3 kb on either side of the *cdtABC* gene cluster [10]. These include a partial ORF encoding a predicted protein with 36% identity to a staphylococcal transposase and a complete ORF encoding a predicted protein 58% identical to a transposon recombinase. These findings raise the

possibility that the *cdtABC* gene cluster was introduced into *H. ducreyi* from another organism.

CDT inhibits cell division and this mechanism may be important in the development of chancroid ulcers or mucosal lesions seen in periodontitis by inhibiting the proliferation of epithelial cells, delaying the healing process (re-epithelialisation) or hampering immunological responses which are important for both healing and immunity. Thus, the clinical picture of infection with CDT-producing strains may be more severe than that with strains lacking the CDT.

In summary, the majority of *H. ducreyi* and *A. actinomycetemcomitans* isolates (82% and 86%, respectively) possess the *cdtABC* genes encoding CDT. The *cdtABC* genes are highly conserved among *H. ducreyi* strains.

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