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Identification of *Actinobacillus actinomycetemcomitans* by Leukotoxin Gene-Specific Hybridization and Polymerase Chain Reaction Assays

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Eleven strains of *Actinobacillus actinomycetemcomitans* isolated from cases of systemic infections, local abscesses, and periodontitis were identified by genetic assays using the leukotoxin gene as the target. We have developed a polymerase chain reaction (PCR) assay, based on the leukotoxin structural gene of this pathogen, which clearly identified all tested strains of *A. actinomycetemcomitans* and separated them from the closely related *Haemophilus aphrophilus* as well as other bacterial species. Furthermore, DNA-DNA hybridization was performed with the cloned partial leukotoxin structural gene (*lktA*) as a probe, which again clearly distinguished *A. actinomycetemcomitans* from *H. aphrophilus*, parts of the normal oral flora, and species harboring RTX (repeats in toxin) family-related cytotoxins. The PCR fragment amplified from the leukotoxin structural gene gave results similar to those given by the cloned leukotoxin gene when used as a probe in hybridization experiments. The hybridization and PCR assays described here are fundamental improvements for the identification of *A. actinomycetemcomitans*.

Actinobacillus actinomycetemcomitans is a fastidious, nonmotile, gram negative bacterium, displaying a coccobacillary or coccoid shape (6). It grows in a CO_2-enriched atmosphere and also anaerobically (6). *A. actinomycetemcomitans* is part of the normal oral flora but can give rise to abscesses, dental plaques, and juvenile periodontitis, as well as endocarditis (6, 7, 13). Therefore, exact identification of this species in clinical samples is most important.

Identification of *A. actinomycetemcomitans* by routine diagnostic assays may be difficult, as strains of this species generally give weak or variable phenotypic expression in conventional characterization assays (17). In our findings for recent clinical isolates of *A. actinomycetemcomitans*, there was an incomplete correlation with catalase production and with other key characteristics such as formation of acid from carbohydrates, properties which are supposed to distinguish *A. actinomycetemcomitans* from the closely related *Haemophilus aphrophilus* and other species (18).

A. actinomycetemcomitans strains produce a leukotoxin that contributes to the killing of human neutrophils and monocytes as part of the pathogenic processes (8, 9). The *A. actinomycetemcomitans* leukotoxin gene cluster has previously been cloned (8–10). Other organisms so far known to harbor members of the RTX (repeats in toxin) family of cytotoxins are *Pasteurella haemolytica* (15), *Actinobacillus pleuropneumoniae* (2, 11), and *Escherichia coli* (4). Leukotoxin activity has not been reported for the closely related *H. aphrophilus*. This virulence factor may therefore represent a means for distinction between *A. actinomycetemcomitans* and *H. aphrophilus*.

The aim of this study was to improve the identification of *A. actinomycetemcomitans* by DNA detection using the leukotoxin gene. The clinical strains included were isolated from cases of systemic infections, local abscesses, and periodontitis. The main specificity challenges in this context comprised the closely related *H. aphrophilus* and *Haemophilus paraphrophilus* (1, 17), other inhabitants of the normal oral flora (6), and other species that harbor cytotoxins with DNA homology to the leukotoxin gene (2, 4, 11, 15).

Here we present the use of the leukotoxin structural gene as a target for DNA hybridization and polymerase chain reaction (PCR) identification assays.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The type strains of *A. actinomycetemcomitans*, *H. aphrophilus*, and *H. paraphrophilus* were included as reference strains in this study (Table 1). In addition, 18 clinical isolates from human disease were included. Their designations and clinical sources are listed in Table 1. All these strains had previously been identified as *A. actinomycetemcomitans*, *H. aphrophilus*, or *H. paraphrophilus* by genetic transformation in our laboratory (reference 18 and unpublished data). Other bacterial strains included in the experiments are listed in Table 1. Strains were grown on chocolate agar plates (Difco) with 5% human blood at 33°C with 5% CO_2.

Construction of plasmid pRH70. The leukotoxin gene cluster of *A. actinomycetemcomitans* Y4 was recently cloned in our laboratory by means of an *E. coli hlyC-hlyA* probe (10). A 7.5-kb *Bam*HI fragment was identified by Southern analysis and was subsequently cloned into pBa, a pBR322 derivative. The resulting recombinant plasmid (pRH70) contained two-thirds of the 5′ region of *lktA* (toxin structural gene) and the complete *lktC* (one of the genes presumably employed in posttranslational modification and secretion of LktA), in addition to 5.1 kb of upstream DNA sequences.

PCRs. Synthetic oligonucleotides (Genetic Designs Inc., Houston, Tex.) TT15 5′-TCGCGAATCAGCTCGCCG-3′ (bases 266 to 282) (8) and TT16 5′-GCTTTGCAAGCTCCT

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TABLE 1. Strains included in the study, clinical source, and RBRs in DNA-DNA hybridizations[a]

Strain (filter DNA source)	Clinical source or condition	Mean RBR (95% confidence interval)[b] with the indicated probe		
		A. actinomycetemcomitans ATCC 9710^T	pRH70 LktA	PCR product LktA
Reference strains				
A. actinomycetemcomitans ATCC 9710^T	Lung abscess	100 (94–106)	100 (94–106)	100 (95–105)
H. aphrophilus				
NCTC 5906^T	Endocarditis	27 (24–30)	6 (5–7)	7 (6–8)
NCTC 5886	Endocarditis	26 (24–28)	5 (4–6)	6 (5–7)
H. paraphrophilus NCTC 10557^T	Paronychia	22 (21–23)	5 (5–5)	4 (3–5)
Clinical isolates[c]				
A. actinomycetemcomitans				
1489/79	Endocarditis	100 (95–105)	98 (95–101)	99 (95–103)
11305/79	Endocarditis	90 (84–96)	96 (87–104)	95 (89–101)
47/87	Unknown	81 (71–91)	93 (83–103)	94 (86–104)
5775/87	Endocarditis	73 (65–81)	95 (91–99)	96 (91–100)
55497/90	Neck abscess	98 (95–101)	93 (90–96)	94 (89–99)
53673/91	Endocarditis	92 (87–97)	90 (85–95)	91 (85–97)
Y4	Periodontitis	103 (100–106)	104 (98–110)	101 (95–107)
FDC 2097	Periodontitis	102 (99–105)	99 (92–106)	103 (100–106)
FDC 511	Periodontitis	95 (91–99)	102 (99–105)	100 (97–103)
SUNY 75	Periodontitis	89 (84–94)	95 (91–99)	96 (92–100)
H. aphrophilus				
EH/79	Endocarditis	20 (18–22)	5	4
IIMO A1/82	Brain abscess	22 (19–25)	6	2
3203/86	Sinusitis	23 (21–25)	4	1
9574/86	Sinusitis	24 (21–27)	6	6
46/87	Unknown	31 (29–33)	4	2
40503/88	Brain abscess	24 (20–28)	5	1
800/89	Brain abscess	23 (20–26)	6	3
24902/91	Sinusitis	21 (19–23)	3	1
Others				
H. influenzae NCTC 8143^T		5	2	1
N. meningitidis 7878		4	3	1
N. gonorrhoeae GC		5	2	3
E. corrodens 31745/80		2	1	1
Pasteurella multocida 646 (pig)		1	6	5
P. haemolytica NVI (serotype 1)		2	5	5
E. coli K-12		0	2	1
P. gingivalis ATCC 33277^T		0	0	0
A. pleuropneumoniae ATCC 33690^T		12 (10–14)	4	5

[a] DNA-DNA hybridizations of *A. actinomycetemcomitans*, *H. aphrophilus* and *H. paraphrophilus*, and species of the oral flora and RTX family were performed with total genomic DNA of *A. actinomycetemcomitans* ATCC 9710^T, plasmid pRH70 containing parts of the *A. actinomycetemcomitans* leukotoxin gene cluster, and a PCR product of the *A. actinomycetemcomitans* leukotoxin gene as radioactively labelled probes.

[b] The means and 95% confidence intervals of the sample means of eight parallels are presented.

[c] The last two figures in these strain designations indicate the year isolated.

CACC-3′ (bases 242 to 260) (8), corresponding to an area in the middle of the *A. actinomycetemcomitans* leukotoxin gene (*lktA*) that displayed the least mutual DNA homology to relevant cytotoxin genes of other species, were made (2, 4, 11, 15). Samples were amplified in a 50-μl reaction mixture containing 10 ng of template DNA, 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, a 200 μM solution of each deoxynucleotide triphosphate (Pharmacia), 0.001% (wt/vol) gelatin, a 0.5 μM solution of each primer, and 0.5 U of Ampli-*Taq* polymerase (Cetus, Norwalk, Conn.). Each sample was amplified in 25 cycles of 30 s at 95°C, 1 min at 65°C, and 1 min at 72°C in a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). Plasmid pRH70 was the positive control template. The resulting positive PCR product was 285 bp. In every set of experiments, negative controls of reaction mixtures with buffer instead of DNA were included. The PCR products were detected by electrophoresis in a 3% Nusieve (FMC Bioproducts, Rockland, Maine)–1% agarose (Sigma) gel. Positive PCR products were checked by hybridization with the labelled PCR product from the template pRH70.

DNA-DNA hybridization. DNA-DNA hybridization was performed essentially as previously described (18). DNA was isolated by the method of Hull et al. (5). The concentration of DNA was adjusted to 200 μg/ml.

(i) Dot blot filters. Eight parallel dots of each single-stranded genomic DNA and a Tris-EDTA buffer control were blotted onto nitrocellulose filters by the Hybri-Dot system (Bethesda Research Laboratories). Filters were stored dry.

(ii) PCR probes. PCR products were prepared for labelling by electroelution from SeaKem GTG agarose (FMC Bioproducts).

(iii) Labelling of probes. Mechanically fragmented genomic

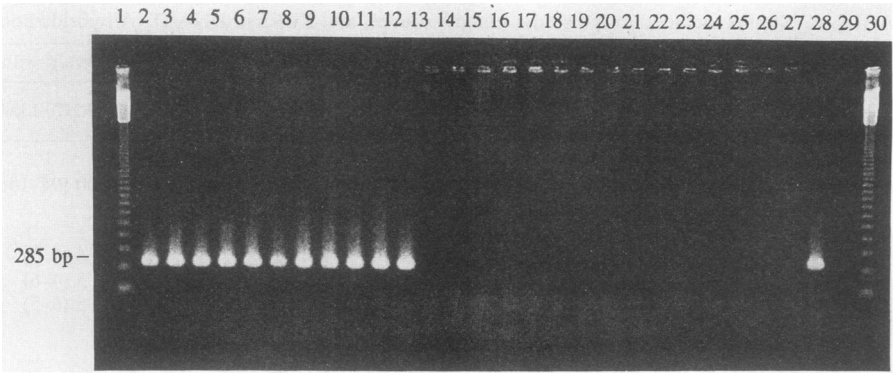

FIG. 1. Results of PCRs with primers based on the *A. actinomycetemcomitans* leukotoxin gene and the following DNA templates: *A. actinomycetemcomitans* ATCC 9710^T (lane 2), 1489/79 (lane 3), 11305/79 (lane 4), 47/87 (lane 5), 5775/87 (lane 6), 55497/90 (lane 7), 53673/91 (lane 8), Y4 (lane 9), FDC 2097 (lane 10), FDC 511 (lane 11), and SUNY 75 (lane 12); *H. aphrophilus* NCTC 5906^T (lane 13), EH/79 (lane 14), 3203/86 (lane 15), 9574/86 (lane 16), 800/89 (lane 17) and 24902/91 (lane 18); *H. paraphrophilus* NCTC 10557^T (lane 19) and 46/87 (lane 20); oral flora species *H. influenzae* NCTC 8143^T (lane 21), *Neisseria meningitidis* 7878 (lane 22), *Eikenella corrodens* 31745/80 (lane 23), and *P. gingivalis* ATCC 33277^T (lane 24); and species harboring RTX-related cytotoxins, i.e., *P. haemolytica* NVI (lane 25), *A. pleuropneumoniae* ATCC 33690^T (lane 26), and *E. coli* K-12 (lane 27). Lane 28 contains a positive control with plasmid pRH70 containing the leukotoxin gene cloned from strain Y4. Lane 29 is a negative buffer control. The molecular size marker in lanes 1 and 30 is a 123-bp ladder (Bethesda Research Laboratories). Amplification products were visualized after electrophoresis in a Nusieve-agarose gel by staining with ethidium bromide.

DNA probes, *Eco*RI-digested pRH70, and PCR probes were labelled with [^{32}P]dCTP to a specific activity of 10^8 cpm/μg of DNA with a random priming labelling kit (Amersham) as recommended by the manufacturer.

(iv) Hybridization. Hybridization was performed for up to 16 h at 65°C in a hybridization oven (Techne). The sodium salt concentrations in the prehybridization and hybridization fluids were 0.1 and 1 M for the total genomic DNA and the PCR probes, respectively. Hybridization washes were performed at 65°C at 0.2× and 2× SSC, respectively (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The results of the dot blot hybridizations were obtained by overnight autoradiography (Hyperfilm MP; Amersham) and scintillation counting (Packard Instruments) of pieces of the nitrocellulose filter cut to standard size with a vacuum-cutting device designed in our laboratory.

(v) Quantitation of the dot blot hybridization reaction. The mean counts per minute for the eight parallel dots minus the control counts per minute of salmon sperm DNA was determined for each of the strains. The mean counts per minute of the autologous strain was defined to represent a DNA homology ratio, or a relative binding ratio (RBR), of 100%. The mean RBR of each strain was calculated by dividing the mean counts per minute of each strain by the mean counts per minute of the autologous reaction mixture and then multiplying by 100. The 95% confidence interval of the sample mean of the RBRs was estimated (Table 1).

RESULTS

Hybridization with the *A. actinomycetemcomitans* total genomic DNA probe. The mean RBRs of strains of *A. actinomycetemcomitans*, *H. aphrophilus*, and other gram-negative species hybridized with a total genomic probe of the *A. actinomycetemcomitans* type strain NCTC 9710 are shown in Table 1. The mean RBRs represent the degree of homology between the probe and the bacterial chromosomes. Generally, the mean RBRs of strains of *A. actinomycetemcomitans* ranged between 73 and 103%, while strains of *H. aphrophilus* had mean RBRs of 20 to 31%.

Hybridization with the *A. actinomycetemcomitans* leukotoxin gene probes. Use of the complete plasmid pRH70 containing the leukotoxin structural gene as a hybridization probe gave mean RBRs of 89 to 100% for strains of *A. actinomycetemcomitans* and RBRs of 3 to 8% for strains of *H. aphrophilus*. Hybridization using the PCR product of the leukotoxin structural gene as a probe gave mean RBRs of 91 to 103% for the *A. actinomycetemcomitans* strains and mean RBRs ranging from 1 to 7% for strains of *H. aphrophilus*.

In all hybridization assays, the strains of *A. actinomycetemcomitans* were compared with a variety of other gram-negative bacterial species representing a relevant spectrum of differential diagnoses in this context. These strains were all well distinguished from *A. actinomycetemcomitans* and *H. aphrophilus*, giving RBRs of 5% or less with both total genomic and leukotoxin-specific probes. The exception to this was *A. pleuropneumoniae*, which had an RBR of 12% with the total genomic probe (Table 1).

Detection of *A. actinomycetemcomitans* by PCR assay. All strains of *A. actinomycetemcomitans* were detected by the appearance of a 285-bp amplification product in the PCR assay (Fig. 1). All strains included in the hybridization assays whose results are reported in Table 1 were included in the PCR assay, although not all are represented in Fig. 1. All PCR products of 285 bp hybridized with the leukotoxin gene-containing plasmids pRH70 and pRH71. At the stringency used, with an annealing temperature of 65°C, there were no PCR products detectable in strains of *H. aphrophilus*, *H. paraphrophilus*, and the other species included in the study. Lowering the annealing temperature to 55°C or less gave a faint band in strains NCTC 5906^T and 9574/86 of *H. aphrophilus* in the expected size range and a larger PCR product (about 460 bp in size) in the strain of *A. pleuropneumoniae*. These products did not hybridize with the leukotoxin gene probe.

DISCUSSION

In this study, genetic identification of *A. actinomycetemcomitans* was achieved by DNA-DNA hybridization and PCR analysis. The information provided represents a means of obtaining a rapid and clear-cut diagnosis of this fastidious

species. Rapid genetic detection of *A. actinomycetemcomitans* may be particularly useful because this species has been recognized as an important marker of periodontal disease (3, 12).

Our aim was rapid and reliable identification of *A. actinomycetemcomitans*. The main specificity challenges in this context were the distinction of *A. actinomycetemcomitans* from the closely related *H. aphrophilus*, from other parts of the normal oral flora, and from other species that harbor cytotoxins with DNA homology to the leukotoxin gene. Genetic transformation assays and hybridization with total genomic probes had previously showed insufficient distinction between *A. actinomycetemcomitans* and *H. aphrophilus* (18).

The PCR assay gave clear-cut identification of the *A. actinomycetemcomitans* strains. Hybridization with the leukotoxin gene-specific probe (plasmid pRH70) gave much better distinction between *A. actinomycetemcomitans* and *H. aphrophilus* than use of total genomic probes (Table 1). This capability to distinguish among *A. actinomycetemcomitans*, *H. aphrophilus*, and other species was even further improved by using the leukotoxin-specific PCR product as a probe. This probe eliminates possible cross-hybridization of leukotoxin gene-flanking sequences which might be conserved between different bacterial species.

Interestingly, all the *A. actinomycetemcomitans* strains reacted similarly in the different assays employed, regardless of their origin. This is in contrast to the variability in leukotoxin production and confirms that expression of leukotoxin is subject to regulation on the transcriptional level or by the stability of the mRNA (14). This might explain why all the strains we tested reacted similarly on the DNA level.

More of a challenge is presented in relation to *H. influenzae*, *Porphyromonas gingivalis*, and other organisms normally present in the human oral flora, as well as other organisms harboring known members of the RTX family of cytotoxins, such as *P. haemolytica* (15) and *E. coli* (4). All of these species displayed very weak or no hybridization with leukotoxin gene probes (Table 1) and were completely negative in the PCR assay (Fig. 1).

Genetic transformation assays based on natural competence have been useful in primary identification of strains of *A. actinomycetemcomitans* (18). Although attempts to use donor DNA derived from an *A. actinomycetemcomitans* strain carrying a chloramphenicol transacetylase gene transcriptionally fused to the leukotoxin gene cluster (*ΔlktAB::cat*) as a selectable marker did improve specificity, the low frequency of transformants recovered with this assay so far hinders its diagnostic usefulness (unpublished results).

Rapid diagnosis of *A. actinomycetemcomitans* is required for detection of this agent in cases of abscesses, endocarditis, and particularly destructive periodontitis (3, 12). Other hybridization assays have been proposed for identification purposes (16), but this is the first gene-specific method reported. The PCR assay presented is quick to perform, while the hybridization assay is inexpensive and simple and can be carried out in any microbiology laboratory, with a hybridization time of as little as 4 h for the PCR probe used here.

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