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Highly Toxic Clone of *Actinobacillus actinomycetemcomitans* in Geographically Widespread Cases of Juvenile Periodontitis in Adolescents of African Origin

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The bacterium *Actinobacillus actinomycetemcomitans* has been implicated in the pathogenesis of juvenile periodontitis as the etiologic agent on the basis of several lines of circumstantial evidence. A matter of extensive debate is whether *A. actinomycetemcomitans* is an exogenous contagious pathogen or an opportunistic pathogen that resides in the normal oral microflora. Here we show evidence of a single clone of *A. actinomycetemcomitans* isolated from multiple patients with juvenile periodontitis in members of families of African origin living in geographically widespread areas. The clone is characterized by a 530-bp deletion in the leukotoxin gene operon, resulting in a significantly increased production of leukotoxin.

Actinobacillus actinomycetemcomitans is a gram-negative rod with a primary habitat in dental plaque in humans. Although the organism may be detected in dental plaque of a significant percentage (17 to 41%) of healthy individuals, constituting a persistent portion of the normal flora (3, 15, 19), several lines of circumstantial evidence suggest that it plays a significant etiologic role in juvenile periodontitis as well as some forms of severe periodontitis in adults (17, 18). However, it is not clear whether disease is induced by an imbalance in the local microbial ecosystem or is due to infection with particularly virulent clones of this bacterial species (4, 10). To evaluate these hypotheses, we previously performed population genetic analyses of two collections of *A. actinomycetemcomitans* isolates, one consisting of a temporally and geographically diverse collection of isolates and the other consisting of isolates from healthy and periodontally diseased Finnish individuals sampled over a limited period of time (6, 14). These studies revealed a very diverse bacterial population, and we found no evidence of especially virulent evolutionary lineages or dissemination of particular clones among patients, as is the case with traditional exogenous pathogens (11–13). On the basis of these observations with isolates from European Caucasians, we concluded that in adult as well as juvenile periodontitis, *A. actinomycetemcomitans* is an opportunistic pathogen belonging to the resident microflora of the human oral cavity.

An important virulence factor of *A. actinomycetemcomitans* is a potent leukotoxin which specifically kills human leukocytes (1). The toxin is encoded by a gene operon which, in addition to the structural gene, includes genes involved in activation and transport of the toxin to the bacterial surface (7, 8). Two genetically closely related isolates examined as part of our previous studies of *A. actinomycetemcomitans* were characterized by a unique restriction fragment length pattern of the leukotoxin gene operon (14). We have shown that this particular pattern is due to a deletion of 530 bp in the promoter region of the leukotoxin gene operon and results in significantly enhanced (10 to 20 times) leukotoxic activity, because a second strong promoter upstream is brought to govern transcription of the operon (2). Both of the isolates were from young African-Americans with juvenile periodontitis. The same version of the leukotoxin gene operon was not detected among more than 200 *A. actinomycetemcomitans* isolates from Caucasians in northern Europe (6, 14).

Juvenile periodontitis is significantly more prevalent among African-Americans (9) and in certain developing countries (5, 16) than among European and American Caucasians. To examine the possibility that particular clones of *A. actinomycetemcomitans* with enhanced leukotoxin activity may be associated with the disease in such populations, we isolated *A. actinomycetemcomitans* from patients with severe juvenile periodontitis in immigrant families living in Denmark and Sweden.

The characteristic 530-bp deletion was detected by PCR (Fig. 1). The PCR primers 5′ TTTCTCCATATTAAATCTC CTTGT 3′ and 5′ CAGATCAAAACCTGATAACAGTATT 3′ were selected from the published *ltx* sequences (2, 8). After a single colony had been boiled for 5 min in 85 μl of water, the PCR was performed at 100 μl with *Taq* DNA polymerase as recommended by the manufacturer (Life Technologies, Roskilde, Denmark) with 30 cycles of a program including denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and polymerization for 2 min at 72°C.

Out of a total of 17 juvenile periodontitis patients examined, strains with the characteristic 530-bp deletion were isolated from 11 subjects (ages, 12 to 21 years) belonging to five families originating from the Cape Verde Islands, Morocco, and Algeria. Among the remaining six subjects, of whom five originated from Morocco and one originated from Kuwait, only two harbored cultivable *A. actinomycetemcomitans*, and the strains isolated from these two patients did not have the deletion and were distinct, as revealed by the analyses described below. The low frequency of the bacterium among these patients may be explained by the fact that they had received treatment prior to sampling. The *A. actinomycetemcomitans* strains with the 530-bp deletion isolated from the 11 subjects

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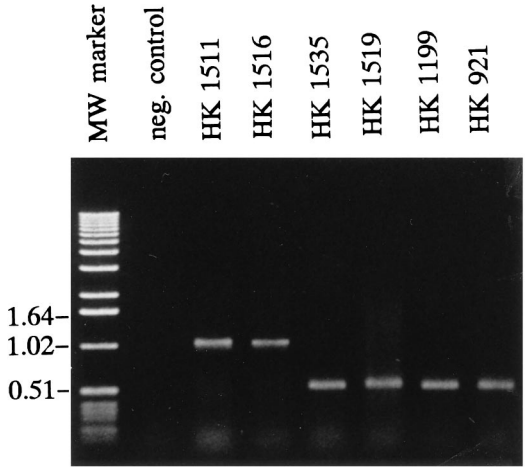

FIG. 1. Detection of the 530-bp deletion in the leukotoxin gene (*ltx*) promoter region. Two primers flanking the deletion were used in a PCR with whole-cell DNA yielding products with sizes of 504 and 1,034 bp in the high- and low-toxicity strains, respectively. The figure includes four isolates with the deletion (HK 921 and HK 1199 from the United States and HK 1519 and HK 1535 isolated from immigrants originating from the Cape Verde Islands and Morocco, respectively) and two strains without the deletion (HK 1516 and HK 1511). MW, molecular weight; neg., negative. Molecular mass markers are in kilobases.

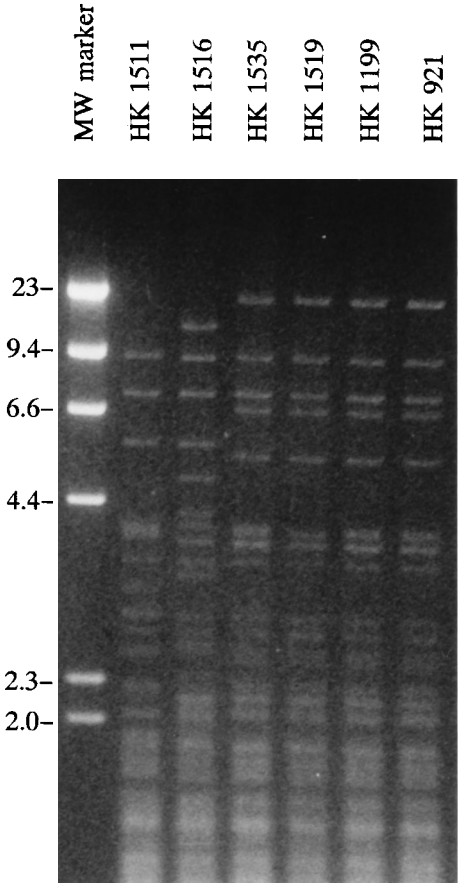

FIG. 2. *A. actinomycetemcomitans* strains with the 530-bp deletion in the *ltx* gene are genetically related. Restriction enzyme analysis of whole-cell DNA of the same six strains as in Fig. 1 digested with *Msp*I showed identical results for all isolates with the deletion and with a pattern not found among strains without the deletion. MW, molecular weight.

were cultivated from subgingival plaque collected from deep periodontal pockets in relation to osseous lesions. Recurrence of the disease was seen in at least two of the patients a few months after conventional treatment, which included surgery and professional tooth cleaning. In two of the families, the mother or father also had a history of juvenile periodontitis. In addition to the two American isolates previously examined, three isolates received from E. T. Lally, University of Pennsylvania, were included in the study. All five American isolates showed the same 530-bp deletion and had been recovered from African-Americans with juvenile periodontitis.

In order to examine whether the 530-bp deletion represents a unique mutational event, i.e., whether strains with the deletion have a common ancestor, a single representative strain from each of the 11 subjects from Denmark and Sweden, together with the 5 North American strains, was subjected to further analyses. Examination of the isolates with antisera specific for the five recognized serotypes (a through e) of *A. actinomycetemcomitans* revealed that they all expressed the serotype b carbohydrate antigen. Multilocus enzyme electrophoresis was performed as previously described (6) and allowed us to compare a total of 10 gene loci encoding intracellular metabolic enzymes, which revealed that 15 of the 16 isolates were identical. The single exceptional isolate from the United States differed in one enzyme. The genetic relationships were further evaluated by restriction enzyme analysis of whole-cell DNA with the two restriction enzymes *Msp*I and *Hae*III. The DNA fingerprints of the 16 isolates were all identical and differed from representative isolates from a collection of more than 200 strains of *A. actinomycetemcomitans* (Fig. 2). These results show that the 16 isolates represent a single and unique clonal type. We previously found that ribotyping with reverse-transcribed 23S and 16S rRNA from *Escherichia coli* as the hybridization probe on Southern blots of *Eco*RI-restricted whole-cell DNA is a very sensitive method to discriminate between individual strains of *A. actinomycetemcomitans* (14). By this technique, we found that the isolates from the Danish and Swedish residents were all identical (Fig. 3). In contrast, each of the five American isolates showed a unique *Eco*RI ribotype. Collectively, these data show that *A. actinomycetemcomitans* isolates characterized by the 530-bp deletion in the leukotoxin gene operon belong to a particular clonal type originating from a common ancestor. The minor differences disclosed by ribotyping suggest that the clone is in the process of evolution while maintaining the characteristic deletion.

The striking finding that all of the strains characterized by the 530-bp deletion were isolated from families originating in Africa suggests that this clone of *A. actinomycetemcomitans* emerged on that continent. The dissemination of the clone has features resembling that of an exogenous pathogen, because our isolates originate from multiple geographically widespread patients with juvenile periodontitis. The characteristic restricted epidemiology suggests that the clone spreads primarily by intrafamilial transmission. A requirement for a particular genetic constitution of the host for colonization combined with social separation of the races may have contributed to this limited dissemination. Further epidemiological studies of *A. actinomycetemcomitans*, especially in Africa, may contribute to the understanding of the epidemiology and pathogenicity of this clonal type.

In conclusion, we suggest that juvenile periodontitis represents two different types of disease with distinct etiologies: one type found worldwide, in which a diversity of *A. actinomycetemcomitans* clones may act as an opportunistic pathogen;

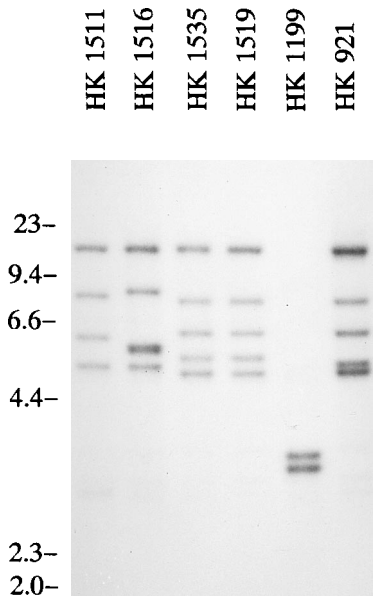

FIG. 3. Highly toxic *A. actinomycetemcomitans* strains are not identical. Southern blots of *Eco*RI-restricted whole-cell DNA hybridized with rRNA sequences (ribotyping) showed identical patterns among *A. actinomycetemcomitans* isolates from Danish and Swedish immigrants of African origin (HK 1519 and HK 1535) and revealed differences among the high-toxicity strains from the United States (HK 921 and HK 1199). The figure also shows two low-toxicity strains (HK 1511 and HK 1516).

and another type, primarily found among adolescents of African origin, in which a particular clonal type of *A. actinomycetemcomitans* serotype b, characterized by the 530-bp deletion, acts as an exogenous pathogen. The observed association of this virulent clone with hosts of African origin may account for the reported increased prevalence of juvenile periodontitis among African-Americans (9) and populations in certain developing countries (5, 16). The suggested differences in etiology and epidemiology have implications for prevention and treatment of the disease; i.e., antibiotics as an adjunct to conventional periodontal therapy may be warranted only when the disease involves this particular virulent and contagious clone.

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