Digital dermatitis (DD), first described in 1974 by Cheli and Mortellaro (R. Cheli and C. Mortellaro, p. 208–213, in Proceedings of the 8th International Conference on Diseases of Cattle, 1974), is a major problem in dairy cows and beef cattle causing significant economic losses worldwide. Lesions are typically found at the volar skin proximal to the heel bulbs. Microscopic examination of biopsies or touch preparations of these lesions revealed a variety of different bacterial morphotypes including significant numbers of spirochetes which often represent the predominant morphotype. We used comparative 16S RNA sequence analysis to determine the diversity and phylogeny of these hitherto unclassified DD spirochetes. Results indicate that those lesions looked at so far contained at least five spirochetal phylotypes, all clustering within the genus Treponema. Phylotype DDKL-4 was nearly identical (99.4% similarity) to that of a nonpathogenic human treponeme, T. phagedenis. Two phylotypes DDKL-3 and DDKL-13 were closely related to those from treponemes commonly found in human periodontitis lesions, i.e., T. denticola and T. vincentii, exhibiting 95 and 98% similarity, respectively. The other two phylotypes, DDKL-12 and DDKL-20, had no close relatives to any cultivable treponemal species but clustered to previously described group IV oral treponemes. Preliminary analysis using in situ hybridization with fluorescently labeled oligonucleotide probes against smears from DD biopsies revealed that from all lesions analyzed so far, T. denticola-like spirochetes were detected in the highest proportion of all spirochetal morphotypes.

Digital dermatitis (DD), first described by Cheli and Mortellaro (6), is an acute or chronic ulcerative epidermitis in cattle that most commonly affects the skin immediately above the coronet between the heel bulbs (4). It is characterized clinically by an erosion of the superficial layers of the epidermis due to the loss of keratin, epithelial hyperplasia and hypertrophy, pain and swelling at the diseased sites, and a typical foul odor. Early lesions often show granulomatous strawberry-like ulcerations, whereas older lesions exhibit a grayish-brown color. DD usually leads to lameness and to a significant decrease in body weight and milk production (4). The disease is found with an incidence varying from 5 to 60% and a prevalence of 2 to 30%, rising to 80% in some herds and causing substantial economic loss in cattle dairies and the meat industry worldwide (38).

Microscopic analysis of specimens taken from DD lesions revealed a variety of different bacterial morphotypes, including gram-negative rods and spirochetes. Spirochetes are often found in great numbers not only in superficial lesions but also in deeper layers of the dermis (5, 11, 25). The etiology of DD is not yet established. However, the presence of high numbers of bacteria, including spirochetes, which apparently invade deeper tissues as well as successful antibiotic therapy strongly suggests a role of bacteria in the etioloLy of the disease (25). So far only a few organisms belonging to the genera Prevotella, Porphyromonas, Fusobacterium, and Bacteroides have been isolated (23). A recent paper reported the isolation of two groups of spirochetes with characteristics most consistent with the genus Treponema (36). However, the exact chemotaxonomic or phylogenetic classification of these organisms has not yet been accomplished.

To facilitate further studies on the etiology of DD and to establish the role of spirochetes, we decided to identify DD spirochetes by comparative 16S RNA sequence analysis. Phylogenetic analysis based on 16S rRNA sequence comparison is well established and has been used successfully to identify yet uncultured pathogenic organisms (26) and endosymbionts (1, 24) or to describe the microbial diversity of complex microbial ecosystems such as mixed infections (8) and environmental microbiota (2, 33, 37). The advantages of using this approach are obvious. It reliably allows a phylogenetically relevant classification, the design of rRNA-targeted oligonucleotide probes or primers for amplification or hybridization assays, and hence a thorough population analysis by fluorescence in situ hybridization (FISH). We therefore established a representative 16S rRNA gene library by cloning in vitro-amplified almost full-length 16S rRNA genes of DD spirochetes into Escherichia coli. Comparative sequence analysis of recombinant clones enabled us to describe five treponemal phylotypes, some of which were closely related to cultivable oral treponemes commonly found in human periodontitis. Preliminary in situ hybridization analysis using fluorescently labeled phylotype-specific oligonucleotide probes revealed a differential distribution of the appropriate organisms in biopsies taken from several DD lesions.

**MATERIALS AND METHODS**

**Light microscopy.** Biopsies (1 by 1 cm) from eight infected dairy cows (Land Brandenburg, Germany) were taken from the plantar sites of bulbs that showed the typical clinical appearance of DD. Biopsies from four cows were pooled and processed for DNA preparation while biopsies from another four cows were used for FISH (see below). Touch preparations of all biopsies were examined by dark-field microscopy for the presence of motile spirochetes or were analyzed by light microscopy after Gram staining. For histologic examination, part of the tissue was fixed in buffered 5% formaldehyde solution, dehydrated, and embedded in paraffin wax. Sections were stained with hematoxylin-eosin (H&E).

**Electron microscopy.** For scanning electron microscopy, sections of the biopsy material were fixed according to the OTOTO method (17), dried with Peldri II...
incubation was continued at 37°C overnight. All further steps were performed as described (32). The final volume of plasmid DNA was 50 pl.

PCR amplification and cloning. Amplification of spirochete 16S rRNA genes (16S rDNA) was performed by using the spirochete-specific forward primer RE-TREP (S'-ccg aat tcg tcv aca ac GT[T/C] TTA AGC ATG CAA GTC-3'; corresponding base positions 1492 to 1509 in Treponema phagedenis) and the universal primer RTU1500 (5'-ccc cgg ttc gca gat GT[T/C] TAC CTT GTT ACG ACT T-3'; corresponding base positions 46 to 63 in 16S rDNA). Underlined lowercase sequences at the 5' end represent the EcoRI, Sall or XmaI, BclIII restriction sites for RE-TREP or RE-RTU1500, respectively. Amplification was done in final volume of 100 pl by adding 1 pl of DNA, 200 pl deoxyribonucleotide triphosphates, buffer (50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 9.0), 2.5 U of Taq polymerase (Gibco BRL, Eggenstein, Germany), and 0.3 pmol each primer. After the addition of mineral oil, PCR was performed in an Omnigene thermal cycler (Hybaid, Teddington, England): DNA was denatured at 93°C for 2 min followed by 30 cycles of 93, 72°C for 1 min each at 1 min at each temperature. The PCR products were purified by agarose electrophoresis and eluted by using the QIAquick gel elution kit (Qiagen, Hilden, Germany). The cloning of amplified 16S rDNA was performed as previously described (8).

Plasmid DNA preparation and sequence analysis. Plasmid DNAs were isolated from 3-ml overnight cultures of E. coli recombinants as previously described (32). The final volume of plasmid DNA was 50 pl in TE buffer (10 mM Tris-HCl, pH 9.0, 1 mM EDTA). The sequences of inserts (16S rDNA) were obtained by using the SequiTherm cycle sequencing kit (BioZym, Oldendorf, Germany) and a LI-COR automated sequencer (model 4000; MWG-Biotech, Ebensburg, Germany) according to the manufacturer's protocols. Sequencing primers used were M13 sequencing primers 40 (5'-GTT TCC CCA CTA GCG AC-3') and 24 (5'-AAC AGC TAT GAC CAT G-3') and universal bacterial primers (19, 39).

Phylogenetic analysis. 16S rDNA sequences were compared with all sequence entries retrievable from current databases (EMBL and GenBank) and unpublished spirochetal sequences (7, 10) by using the sequence analysis program DNASIS. (DNASIS was purchased from medium volume (10 ml) were fixed in 5% paraformaldehyde in PBS and washed with PBS. Heat-denatured probe RTU1500 was spotted onto glass slides with 12 separate wells coated with 0.01% bovine serum albumin and 0.1% gelatin. The slides were air-dried and dehydrated in 50, 80, and 99% ethanol for 3 min each.

Oligonucleotides were enzymatically labeled at their 3' ends with Cy3 (indentocyanin)-dUTP (Amersham, Braunschweig, Germany) or fluorescein-dUTP (Boehringer, Mannheim, Germany) by using terminal transferase according to the digoxigenin-labeling protocols (Boehringer, Mannheim, Germany).

An aliquot (10 µl) of hybridization solution (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.01% SDS) containing 50 ng of the appropriate oligonucleotide probe was applied to each well of the slides and incubated in a humid chamber in the dark at 40°C for 2.5 h. The stringency was adjusted by adding formamide in different concentrations to the hybridization solution. The slides were rinsed with distilled water, air dried, and examined under oil immersion with a 100× Neofluar objective on a Zeiss Axioskop (Jena, Germany) equipped with a high pressure mercury bulb (HBO 50) and filter sets 487915 (BP 540/12, FT580, LP900) and 487909 (BP 490-490, FT510, LP 515). Photomicrographs were taken on a Kodak Ektachrome HC 400 film.

RESULTS

Morphology and histology of DD lesions. Biopsies were taken from diseased cows showing characteristic wet, exudative lesions as depicted in Fig. 1A. The ulcers were characterized by a dark red proliferative area surrounded by a white epithelial margin extending from the skin to the horn of the heel between two claws. H&E-stained sections revealed histologic features of an acute inflammation characterized by necrosis of the superficial layers, hyper- or parakeratosis, and spongiosis changes. In advanced lesions epidermal layers were completely absent with dermal papillae reaching to the surface thus producing the typical granulomatous appearance. Capillaries were hyperemic with enlarged nuclei of the endothelial cells. Occasionally local hemorrhages occurred. The periphery of the papillary blood vessels was characterized by an infiltrate consisting of mainly polymorphonuclear leucocytes with lower numbers of macrophages or lymphocytes and only a few plasma cells (Fig. 1B). Electron microscopy revealed huge numbers of spirochetes that resulted from keratolytic activity (Fig. 1C). Electron microscopy revealed huge numbers of spirochetes that resulted from keratolytic activity (Fig. 1C).
FIG. 1. Clinicopathological appearance of DD lesions. (A) Typical lesions of DD with circumscribed superficial ulcerations of the skin proximal to the bovine coronary band. (B) Histopathology (H&E staining) of DD lesions showing necrotic superficial epidermal layers and inflammatory infiltration of the dermal papillae. (C) Representative transmission electron micrograph showing numerous spirochetes in an advanced stage of a typical DD lesion.
DD biopsies collected from four animals, inserts of 20 randomly chosen recombinant clones were sequenced. Comparative sequence analysis showed that all recombinants except one that exhibited *E. coli* 16S rDNA sequences contained spirochetal rDNA inserts. Phylogenetic analysis revealed five phylogenotypes: DDKL-3, DDKL-4, DDKL-12, DDKL-13, and DDKL-20. For DDKL-3, DDKL-4, and DDKL-13 nearly complete 16S rDNA sequences (1,313 to 1,428 bases) were determined. A phylogenetic tree (Fig. 3) based upon the comparison of 580 bases (corresponding to positions 109 to 677 in *E. coli* 16S rRNA) shows the relationship of DD spirochetes to known spirochetes. Phylogenotype DDKL-4, represented by four clones, was nearly identical (99.4% similarity) to the sequence of a non-pathogenic human treponeme, *T. phagedenis* strain K. DDKL-3 was represented by nine clones and showed sequence similarity of approximately 95% to an oral treponeme, *T. denticola*. Phylogenotype DDKL-13 represented by only one clone revealed 98% similarity to an oral treponeme, *T. vincentii*, and was nearly identical (2-base-pair mismatch) to phylogenotype sequence NZM3142 previously detected in a subgingival plaque sample of a patient with destructive periodontitis (8). DDKL-12 and DDKL-20, represented by one clone each, had no close relatives among known treponemes, but they clustered to previously described group IV treponemes (8). Two clones that contained chimeric sequences composed of DDKL-3 and DDKL-4 were identified by the program CHECK-CHIMERA (20).

**Whole-cell in situ hybridization for direct identification of treponeme phylogenotypes from DD lesions.** To identify and enumerate DD treponemes characterized by comparative sequence analysis directly in material from DD lesions, smears of another four biopsy specimens were processed for in situ hybridization. Oligonucleotide probes DDK4 specific for both *T. phagedenis* and phylogenotype DDKL-4, DDK3 specific for phylogenotype DDKL-3 (a close relative of *T. denticola*), and Tre I for phylogenotype DDKL-13 were used after labeling with Cy3 or fluorescein. The probe Tre I was initially designed for detection of all group I oral treponemes including *T. vincentii* (22). The specificity of each oligonucleotide was tested by dot blot hybridization using recombinant plasmid DNA as a target. As shown in Fig. 4, probe DDK4 and Tre I detected larger treponemes, while DDK3 identified smaller cells with tight spirals. In all samples examined so far the DDKL-3 (*T. denticola*-like) phylogenotype was detected most frequently, whereas other phylogenotypes were present in only small numbers. Only a minor pro-

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**FIG. 2.** Microscopic appearance of DD spirochetes. (A) Gram-stained touch preparation of infected tissue. (B) Scanning electron micrograph showing different spirochetal morphotypes (1 and 2) with diameters of 0.17 and 0.27 μm, respectively.

**FIG. 3.** Phylogeny of DD spirochetes. 16S rRNA-based phylogenetic tree showing the relationships of DD spirochetes (DDKL-) to other spirochetes, using *Escherichia coli* as an outgroup. The tree was based on 100 bootstrap samplings of partial sequences (about 580 bases) by using the Jukes & Cantor (16) correction and the neighbor-joining (31) method. The scale bar represents 10% difference in nucleotide sequences as determined by taking the sum of the length of the horizontal lines connecting two sequences.
FIG. 4. FISH. Different treponemal phylotypes were detected by using fluorescently labeled oligonucleotide probes. Phase-contrast (left) and epifluorescence photomicrographs (right) of the same field are shown for each preparation. (A) Probe Tre I, labeled with fluorescein-ddUTP, was used for the detection of *T. vincentii*-related organisms. Probes DDK4 and DDK3 labeled with Cy3-dCTP were used for the detection of *T. phagedenis* (panel B) or *T. denticola*-like (panel C) phylotypes, respectively.
portion of spirochetal morphotypes were not detected by the three probes used.

DISCUSSION

As shown by light and electron microscopy, spirochetes were present in large numbers in touch preparations or tissue sections of lesions typical for DD in cattle. They far outnumber other bacterial morphotypes, and their presence is always associated with necrotic changes of the infected tissue, suggesting a possible etiologic role of these organisms. Different spirochetal morphotypes varying in length, width, and number of endoflagella have been described before (11, 14, 25, 38). These observations correspond well with a recent report on the successful isolation of two distinct spirochetal phenotypes from papillomatous digital dermatitis and interdigital dermatitis, suggesting that these organisms belonged to the genus Treponema (36). However, despite the description of some phenotypic traits the exact taxonomic classification for these isolates has not been determined.

In contrast, the phylogenetic approach using comparative 16S rRNA sequence analysis not only provided a reliable taxonomic classification but also allowed the design of diagnostic hybridization probes useful for direct visualization of novel organisms and further epidemiologic studies. Although complete 16S rRNA sequences will be necessary for determinative phylogenetic description, the comparison of partial sequences comprising all relevant spirochetal signature sequences has been shown to be sufficient for the description of treponemal diversity within a complex microbial population of a subgingival plaque sample (8). The validity of this approach and the accuracy of the sequence analysis have recently been documented by successful isolation and subsequent analysis of the entire 16S rRNA gene sequence from a number of hitherto uncultured human oral treponemes that were previously identified by molecular genetic techniques (40). These data indicated that a critical analysis of partial 16S rRNA sequences provides appropriate phylogenetic information. However, it is crucial to carefully check all sequence entries for the presence of chimeric sequences (18, 30). By using the CHECK_CHIMERA program, we identified two chimeras composed of two parental treponeme sequences that were cross-amplified at positions corresponding to bases 310 to 360 and 660 to 760 in E. coli 16S rRNA. Our data clearly indicate that spirochetes associated with DD in cattle belong to the genus Treponema, thus corroborating prior observations that suggested this taxonomic affiliation on the basis of phenotypic traits, e.g., size, insertion or number of periplasmic flagella, antigenic characteristics and enzymatic activity (14, 36). A report on a positive B. burgdorferi serology in DD cattle, suggesting that this organism might be implicated in DD, is explained either by the presence of cross-reacting antibodies or by the fact that animals included in these studies were confected with B. burgdorferi (3). Our study, however, clearly shows that spirochetes present in DD lesions are not borreliae but belong without any doubt to the genus Treponema.

To our surprise, two phylotypes, DDKL-3 and DDKL-13, were closely related to oral treponemes, T. denticola (95% similarity) and T. vincentii (98% similarity), commonly associated with human periodontal infections. This striking relationship to human oral treponemes is underscored by the observation that monoclonal antibody H9-2 directed against a 37-kDa endoflagellar sheath protein of T. pallidum (21) and used in the past to identify yet uncultured invasive oral treponemes (pathogen-related oral spirochetes [PROS]) in patients with severe periodontitis or acute necrotizing ulcerative gingivitis (27, 28) cross-reacted with treponemes found within DD lesions (data not shown). Recently we showed that PROS constitute a heterogenous group of spirochetes that belong to phylogenetic group I of oral treponemes, a group that includes T. vincentii and related organisms (9). Phylotypes DDKL-12 and DDKL-20 do not bear close resemblance to any known treponeme. They cluster, however, to group IV of oral treponemes, a taxon that has been defined by comparative 16S rRNA analysis (8).

Mere numeric association suggested that phylotype DDKL-3, a close relative of the well-established oral pathogen T. denticola (12), might play a role in the pathogenesis of DD lesions. In contrast to other DD treponemes, T. denticola-like organisms occur in high proportions. However, the number of DD specimens analyzed so far is too small. A detailed epidemiologic study is needed. Another criterion for assessing their pathogenic role would be to identify tissue-invasive spirochetes commonly found in chronic DD lesions. We are currently approaching this problem by using fluorescence-labeled oligonucleotide probes in situ hybridization experiments. However, to finally determine the etiologic relevance of single species in mixed infections it will be necessary to study bacterial interactions. Such an interaction has been shown for heel abercesses in sheep, where the causative organisms Fusobacterium necrophorum and Actinomyces ( Corynebacterium) pyogenes occur in different proportions but complement each other's ability to stimulate growth and to attack phagocytes (29). The virulence determinants of bacteria present in DD lesions have yet to be investigated. Molecular epidemiologic studies using FISH may guide us to successfully design such experiments aimed at elucidating microbial interactions in mixed infections, an area of increasing importance for both veterinary and human medicine (34). As compared to periodontal infections, the analysis of these interactions in DD might be easier due to the relatively small number of bacterial species associated with this disease. Hence, it seems possible to use DD as a model system for studying the pathogenesis and prevention of human periodontitis.

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REFERENCES

10. Dewhirst, F. E. Unpublished results.


