Fluorescence *in situ* hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions

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Fluorescence *in situ* hybridization (FISH) was performed on sections of plastic-embedded tissue using 16S rRNA-directed oligonucleotide probes to visualize uncultured treponemes in skin biopsies of cows with digital dermatitis. Plastic as embedding material allowed sectioning of hard and soft tissue with a defined thickness, avoiding the risk of dragging bacteria into the tissue while sectioning. Furthermore, it provided a good signal-to-noise ratio. Using this method the spatial distribution of three different bacterial phylotypes was visualized simultaneously within the tissue. Whereas debris covering the ulcers contained a mixture of different micro-organisms, a layering of certain treponemal phylotypes was observed deeper in the epidermis. Confocal laser scanning microscopy and subsequent three-dimensional reconstruction of series of optical sections confirmed that the treponemes migrated intercellularly around the cells, most of them directed towards the dermis. *In situ* hybridization on tissue embedded in plastic proved to be a useful method to study mixed bacterial infections since it combines excellent histological conservation of tissue with identification of bacterial species by simultaneous use of probes labelled with different fluorescent dyes. This technique may have implications for *in situ* detection, identification and localization of micro-organisms in veterinary as well as in human medicine.

Keywords: treponemes, tissue sections, *in situ* hybridization, FISH

INTRODUCTION

In situ hybridization (ISH) has been proven to be a useful method for detection and identification of bacteria within their natural environments. It has been used to visualize uncultured micro-organisms to estimate their size, number and morphology (Amann *et al.*, 1991; DeLong *et al.*, 1989). Recently, it was applied to identify spirochaetes from digital dermatitis (DD) lesions in cattle (Choi *et al.*, 1997). DD, first described by Cheli & Mortellaro (1974), is an ulcerative epidermitis typically found at the plantar skin proximal to the heel bulbs of

dairy cows and beef cattle. Clinically, it is characterized by wet, exudative and typically malodorous lesions with pain and swelling at the diseased site. DD causes substantial economic losses worldwide (Blowey & Sharp, 1988; Weaver *et al.*, 1993) due to lameness, reduced fertility and significant decrease in body weight and milk production.

Microscopic analysis of touch preparations of DD lesions typically shows spirochaetes as the predominant morphotype, suggesting a possible aetiological role for these organisms (Blowey *et al.*, 1992; Done *et al.*, 1993; Read *et al.*, 1992). Recently, comparative 16S rDNA analysis showed that these as yet unclassified DD spirochaetes are closely related to cultivable as well as to uncultured oral treponemes (Choi *et al.*, 1997). Using 16S rRNA-directed oligonucleotide probes in ISH of

Abbreviations: DD, digital dermatitis; CLSM, confocal laser scanning microscopy; FISH, fluorescence *in situ* hybridization; H&E, haematoxylin/eosin; ISH, *in situ* hybridization.

touch preparations, *Treponema denticola*-like spirochaetes were detected in the highest proportions among all treponemal species. However, it is not clear whether these organisms are simply commensals proliferating in the anaerobic milieu of a skin ulcer or actively involved in primary inflammation. Using Warthin–Starry (Blowey *et al.*, 1994) or silver staining (Elias & Bosma, 1987), DD spirochaetes have been detected within tissue sections. Light and transmission electron microscopy have indeed shown numerous spirochaetes between or inside necrotic cells within the stratum corneum and stratum granulosum, but again species identification was not possible by these methods.

The specific aim of this study was to identify treponemes directly within the epidermis and to visualize their spatial distribution using fluorescence *in situ* hybridization (FISH).

METHODS

Bacterial strains. The following bacterial strains were used as controls to optimize the ISH conditions: *Treponema vincentii* (ATCC 33580 and RITZ A), *Treponema denticola* (ATCC 33521), *Treponema pectinovorum* (ATCC 33768), *Treponema socranskii* subsp. *buccale* (ATCC 35534), *Treponema socranskii* subsp. *socranskii* (ATCC 35536), *Treponema maltophilum* (Wyss *et al.*, 1996) and *Treponema phagedenis* subsp. *reiteri* (kindly provided by B. Wilske, Munich, Germany).

Processing of tissue specimens. Biopsies (1 × 1 cm) were taken from typical DD lesions of 10 infected dairy cows from 10 different farms (Land Brandenburg, Germany) and transported to the laboratory immediately. All of the following steps were performed at a temperature of 4 °C. The tissue was cut into slices with a maximum thickness of 0.3 cm and fixed with 3.7% (v/v) formaldehyde in PBS (pH 7.4) for at least 3 h. The embedding procedure, utilizing cold polymerizing resin (Technovit 8100, Kulzer), was performed according to the manufacturer's instructions. Briefly, the specimens were washed overnight in PBS containing 6.8% (w/v) sucrose, dehydrated in acetone for 1 h and infiltrated with the plastic solution (Technovit 8100 base-liquid with hardener I) for at least 6 h. After adding hardener II, the biopsies were aligned properly in the wells of histoform S (Kulzer), sealed with cover foil and placed in the refrigerator. After polymerization, histoblocks were mounted on the specimen using Technovit 3040. The blocks were stored at 4 °C prior to sectioning. Formaldehyde-fixed biopsies were routinely embedded in

paraffin in parallel. After dehydration in a graded alcohol series, they were transferred to paraffin via xylene and embedded in tissue-tec moulds (Kulzer).

Sectioning and light microscopy. Blocks were sectioned on a rotary microtome (Medim, Type DDM 0036) using steel knives with hard metal edges. Tissue sections (4 µm) were straightened on sterile water, placed on silanized slides (Super Frost, Medim; 3-aminopropyltrimethoxysilane, Sigma) and stored at 4 °C.

For histological evaluation, tissue sections were stained with haematoxylin/eosin (H&E) and examined using an Axioskop microscope (Zeiss).

Oligonucleotide probes. Phylotype-specific oligonucleotide probe DDK4 (Table 1), corresponding to position 1246–1267 in *Escherichia coli* 16S rRNA, was designed to be complementary to the sequence of a clone derived from a 16S rDNA gene library of DD spirochaetes reported previously (Choi *et al.*, 1997). Group-specific probes TRE I, II and IV (Table 1) were designed after comparative sequence analysis of clones from a 16S rDNA library representing cultivable and as yet uncultured oral treponemes associated with periodontal disease (Choi *et al.*, 1994; Moter *et al.*, 1998). To assess specificity, probe sequences were compared with those of all 16S rRNA entries in the EMBL and GenBank databases (July 1997) using the Husar program package (DKFZ, Heidelberg, Germany). In addition, all oligonucleotide probes were tested by dot-blot hybridizations against PCR-amplified 16S rDNA from 34 bacteria. These controls included sequences from clones of a gene bank of uncultured oral treponemes derived from a 16S rDNA library, cultivable *Treponema* species and oral pathogens as described previously (Moter *et al.*, 1998; Choi *et al.*, 1997). Cultivable representatives of these spirochaetal groups targeted by probes TRE I, II, IV and DDK4 are *T. vincentii*, *T. denticola*, *T. maltophilum* and *T. phagedenis*, respectively (Table 1). EUB 338 (5' GCTGCCTCCCGTAG-GAGT), specific for the domain *Bacteria*, was used to visualize the entire bacterial population in the specimens (Amann *et al.*, 1990). End-labelling (3') of oligonucleotide probes with Cy3-dCTP or Cy5-dCTP [5-aminopropargyl-2'-deoxycytidine 5'-triphosphate coupled to Cy3 (indocarbocyanine) or Cy5 fluorescent dye, Amersham] was performed enzymically using terminal transferase (Boehringer Mannheim). Fluorescein-labelled (5') probes were obtained from Tib Molbiol (Berlin).

FISH. Hybridization buffer contained 0.9 M NaCl, 20 mM Tris/HCl, pH 7.3, and 0.01% SDS. The stringency was adjusted by varying the formamide concentration from 0 to 30% (v/v) depending on the oligonucleotide probe used. To assess specificity of the probes, all available *Treponema* strains served as controls, using fixed cells (Moter *et al.*, 1998) or

Table 1. Treponeme-specific oligonucleotide probes used for FISH

Phylogenetic group of oral treponemes	Oligonucleotide probe	Sequence (5' → 3')	Reference	Cultivable representative
I	TRE I	ACGCAAGCTCATCCTCAAG	Choi <i>et al.</i> (1997)	<i>T. vincentii</i>
II	TRE II	GCTCCTTTTCCTCATTTACCTTTAT	Moter <i>et al.</i> (1998)	<i>T. denticola</i>
IV	TRE IV	CGGTCACATTCGGTATTACCTACT	Moter <i>et al.</i> (1998)	<i>T. maltophilum</i>
–	DDK4	ACAGTCTCGCTTCACCTTTGTAG	Choi <i>et al.</i> (1997)	<i>T. phagedenis</i>

sections of plastic-embedded cultures. Control slides with fixed treponeme cultures were included in every hybridization experiment with tissue sections. Of all 10 biopsies, at least three sections were investigated in three different hybridization experiments. Pre-warmed hybridization solution (20 µl) was mixed with approximately 5 pmol of the appropriate oligonucleotide probe and carefully applied to the tissue sections. After incubation for 3.5 h in a dark humid chamber at 46 °C, slides were washed with pre-warmed buffer (20 mM Tris/HCl, pH 7.4, 0.01% SDS) for 15 min at 46 °C. The NaCl concentration was adjusted from 0.112 to 0.45 M, according to the formamide concentration in the hybridization solution. Slides were then rinsed with distilled water, air-dried in the dark and mounted with Citifluor AF 1 (Chemical laboratory of the University of Kent). Paraffin sections were dewaxed in xylene for 15 min and rehydrated in 96, 70 and 50% (v/v) ethanol (5 min each) prior to hybridization.

Epifluorescent microscopy and CLSM. A Zeiss Axioskop epifluorescence microscope was used to view the bacteria in sections processed for FISH. The microscope was equipped with a 50 W high pressure mercury lamp (HBO50, Osram) and 10×, 40× and 100× objectives (Zeiss). Narrow band Filter sets HQ-F41-007 and HQ-F41-001 (AHF) were used to analyse FITC and Cy3 signals, respectively. Photomicrographs were taken on Kodak Ektachrome HC 400 film. A Zeiss LSM 410 scanning confocal microscope equipped with an Ar-ion laser (488 nm) and two HeNe lasers (543 and 633 nm) was used to record optical sections. Image processing was performed with a standard software package delivered with the instrument (Zeiss LSM version 3.95). Reconstructed and processed images were reproduced on slide film (Kodak Professional, HC 100).

RESULTS

Histology of DD lesions

H&E staining of the skin sections revealed signs of acute inflammation with a highly hyperplastic epidermis and pronounced rete-ridge formation. The corneal, granulosal and superficial spinosal cell layers were eroded and the tips of the dermal papillae were ulcerated (Fig. 1a). Erythrocytes, leukocytes and debris of destroyed keratinocytes were mixed with particulate material and micro-organisms, and covered the lesion as a crust.

Embedding procedure and hybridization conditions

Skin biopsies were embedded into the cold polymerizing resin Technovit 8100. The entire procedure required 2 d, but only 1 h of actual laboratory work. Biopsies up to 10 × 5 × 3 mm could be processed easily. Sectioning of soft and hard tissue was equally feasible with exactly defined thickness ranging from 1 to 30 µm. Conditions for conventional FISH protocols for touch preparations were easily adapted to tissue FISH. Stringent hybridization conditions were determined for each probe using sections of plastic-embedded or fixed treponeme cultures (data not shown). Optimal

hybridization stringency was achieved by adding formamide to the hybridization buffer: 10% final concentration for TRE I and IV, 20% for TRE II and DDK4. However, as no significant reduction in signal intensity for TRE I and IV was observed using 20% formamide, all hybridizations were performed at this concentration. Resin sections did not require any further treatment, such as etching or dehydration, before applying the hybridization solution. When silanized slides were used, there was no need for coverslips or sealing of the samples because the hybridization solution remained on the plastic sections without floating off the slide. Tissue sections could be stored for up to 6 months without any significant signal reduction. After hybridization, the slides could be examined within 1 week. The preparations were analysed equally well by epifluorescent microscopy and confocal laser scanning microscopy (CLSM). However, epifluorescence microscopy allowed a better orientation within the tissue, whereas CLSM allowed three-dimensional reconstruction (data not shown) and exact localization of the micro-organisms outside the cells.

Direct visualization of bacteria by FISH

To identify tissue-invasive treponemes possibly involved in the pathogenesis of disease, the spatial distribution of spirochaetes within the epidermis of DD ulcers was investigated using FISH on sections of tissue embedded in the cold polymerizing resin Technovit 8100. Excellent morphological resolution provided exact histological orientation within the tissue. A high signal-to-noise ratio and the slight autofluorescent background allowed location of bacteria within the epidermis. From all 10 biopsies, three sections each were used in independent experiments. Hybridization with EUB 338 showed a variety of different bacterial morphotypes mixed with autofluorescent erythrocytes in the debris covering the ulcer. Simultaneous hybridization with probe DDK4 revealed large numbers of spirochaetes in this area. While most of the EUB 338-stained bacteria were visible in the debris on the surface of the ulcer, the majority of treponemes were located within the tissue (Fig. 1b). Focusing on the spinosal cell layers of the epidermis, DDK4-positive bacteria appeared to be in bundles around the cells and directed towards the dermis. No spiral-shaped and long stretched bacteria were visualized in this region using EUB 338 (Fig. 1c). CLSM confirmed that the treponemes were indeed located around the cells (Fig. 1d), whereas no bacteria were visible within cells.

Three biopsies were further analysed by using different combinations of specific oligonucleotide probes simultaneously, revealing a stratification of different treponemal phylotypes within the epidermis (Fig. 2). Group II treponemes (*T. denticola*-related) were only detected in the very superficial layers (Fig. 2b), whereas TRE IV- (data not shown), TRE I- and DDK4-positive

Fig. 1. For legend see facing page.

treponemes migrated deep into the stratum spinosum (Fig. 1e). Using EUB 338, an additional distinct morphotype of long stretched bacteria, not detectable with any of the other probes used here, was visualized deep in the epidermis next to the dermal papillae.

The same FISH protocol used on paraffin sections did not give satisfactory results. The bacteria appeared rather hazy and hard to distinguish in the high background of non-specifically stained material (data not shown).

Fig. 1. Histopathology and FISH of tissue sections from DD lesions. (a) H&E staining showing typical histology with necrotic superficial epidermal layers. Bar, 50 μ m. (b) FISH of the same area using universal probe EUB 338_{FITC} (green) and DDK 4_{Cy3} (yellow) exhibits stratification of fluorescence signals, localizing EUB 338-positive bacteria predominantly in the debris and DDK4-positive treponemes in the stratum corneum and stratum spinosum. Bar, 50 μ m. (c) Using the FITC filter set, no rods or cocci were detected by EUB 338. In the upper part of the micrograph large numbers of micro-organisms are visible. Bar, 10 μ m. (d, e) Deeper in the tissue single spirochaetes are visible between cells directed towards the dermis. CLSM of simultaneous hybridization with TRE I_{Cy5} (green) and DDK 4_{Cy3} (red) shows both phylotypes migrating around the cells deep (d) and even deeper (e) within the tissue (e). Bars, 5 μ m. (f) EUB 338-stained bacteria migrating deepest into the tissue are visible close to a dermal papilla. Bar, 5 μ m. The diagram below (e) and (f) shows the relationship between the various parts of the figure.

Fig. 2. Spatial distribution of treponemes within the epidermis as shown by CLSM. Identical microscopic fields after simultaneous hybridization with (a) EUB 338_{FITC}, (b) TRE II_{Cy3} and (c) TRE I_{Cy5} are shown. Note the stratification of the different phylotypes. Bars, 15 μ m. The diagram on the left highlights the features shown in (a)–(c).

DISCUSSION

FISH is a powerful technique for the investigation of mixed populations of micro-organisms in their natural environments. It has been used to visualize cultivable and as yet uncultured bacteria in activated sludge (Wagner *et al.*, 1994a, b, 1995) and in clinical and pathological samples (McNicol & Farquharson, 1997). It enables phylogenetic identification using 16S rRNA-directed oligonucleotide probes in addition to providing information on size, morphology and numbers of bacteria. The method has been further improved by CLSM, allowing optical sectioning, three-dimensional reconstruction and therefore exact localization and observation of the spatial distribution of micro-organisms (Amann *et al.*, 1996; MacNaughton *et al.*, 1996; Manz *et al.*, 1995; Surmann *et al.*, 1996; Wilson, 1989; Wright & Schatten, 1991).

In the past, visualization of bacteria by FISH within biopsy sections was hampered by high levels of non-specific background staining in paraffin-embedded tissue (Licht *et al.*, 1996; Poulsen *et al.*, 1994). Better results have been reported using cryostat sections; however, a low signal-to-noise ratio did not allow the exact histological localization and simultaneous detailed morphological description of the target organisms (Forsgren *et al.*, 1994, 1996). Bacteria have been successfully detected in tissue sections by using radioactively labelled (Ward *et al.*, 1996), digoxigenin-labelled or sulphonated probes with subsequent immunoenzymic detection, although all three methods require extensive prehybridization, blocking, washing and detection steps. Therefore, ISH has been considered a laborious, time-consuming method, unsuitable for diagnostic investigations (Theil *et al.*, 1996). Furthermore, additional pretreatment of the sections with Proteinase K is often necessary to allow binding of the oligonucleotide probes (Arnoldi *et al.*, 1992; Karttunen *et al.*, 1996; Yoshii *et al.*, 1995). In some specimens only weak signals have been obtained even after proteinase digestion (Yoshii *et al.*, 1995) and high concentrations of Proteinase K have resulted in tissue destruction (Karttunen *et al.*, 1996). Since we were interested in elucidating the aetiology of mixed infections, such as DD or human periodontitis, the use of oligonucleotide probes labelled with different fluorescent dyes would allow simultaneous visualization of more than one bacterial species within inflamed tissue. Recent comparative 16S rRNA analysis has allowed determination of the phylogeny of mainly uncultured spirochaetes in both DD and human periodontitis (Choi *et al.*, 1997, 1994; Rijpkema *et al.*, 1997). Light microscopy showed that spirochaetes were the predominant morphotype in both diseases. However, as these diseases are mixed bacterial infections, the mere presence of a given species or morphotype does not prove its aetiological relevance.

Among the cultivable oral species, *T. denticola*, a member of the group II treponemes, is thought to play a mayor role in initiating and maintaining periodontitis. Extensive *in vitro* studies have been performed to

analyse its virulence factors, suggesting the invasive potential of *T. denticola* (Arakawa & Kuramitsu, 1994; Baehni *et al.*, 1992; Ellen *et al.*, 1994; Grenier *et al.*, 1990; Rosen *et al.*, 1994, 1995; Syed *et al.*, 1993; Uitto *et al.*, 1995). Closely related bacteria were detected in smears of DD lesions using 16S rRNA analysis. In the study reported here, FISH gave positive signals with TRE II only in detritus and superficial layers of the stratum spinosum of DD ulcers, suggesting that *T. denticola*-related treponemes were not the key pathogens in the cases investigated. However, treponemes residing deep within the tissue stained positive with probes DDK 4, TRE I and IV, the latter two having been originally designed for detection of oral treponemes (Moter *et al.*, 1998). The morphotype that migrated deepest into the epidermis only stained positive with EUB 338, but failed to react with any of the spirochaete probes used here. This might be due to the fact that the available probes do not enable detection of all spirochaetes, underlining the considerable diversity of this group. In addition, at present it cannot be excluded that these organisms may be fusobacteria, known to co-aggregate with oral treponemes (Kolenbrander *et al.*, 1995) and further FISH studies are required to address this question.

In conclusion, fluorescence *in situ* hybridization on sections of plastic-embedded tissue appears to be of potential value in elucidating the aetiopathogenesis of mixed bacterial infections and in allowing rapid diagnosis in infections in animals and man. In particular, FISH on tissue sections of DD-infected cows showed a spatial distribution of as yet uncultured treponemes within the epidermis. Since tissue invasion is considered a major virulence trait, some treponemes appear to have a role in the pathogenesis of this disease.

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