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Abstract

Digital dermatitis (DD) of cattle leads to lameness and a decrease of milk production and is responsible for major economic losses worldwide. Although a bacterial aetiology is generally accepted, it still is unclear which microorganisms cause and/or maintain the disease. Recently, a previously undiscovered bacterial species, Guggenheimella bovis, has been isolated from the front of two DD lesions in Swiss cattle and suggested as a potential pathogen. The aims of the present study were to determine the prevalence of G. bovis in 58 German cows suffering from DD via dot blot hybridization, and to analyse the spatial distribution of G. bovis within the affected tissue by fluorescence in situ hybridization (FISH). A species-specific probe, GUBO1, was designed and evaluated. In none of the 58 samples Guggenheimella could be detected, while cultured G. bovis was reliably identified by GUBO1. Further FISH experiments were carried out on two additional biopsies of Swiss cattle tested positive for G. bovis by quantitative PCR and permitted visualization of the newly discovered bacteria in situ. In these biopsies G. bovis proved to be tissue invasive forming characteristic spherical microcolonies not only within the bacterial biofilm but also in seemingly unaffected parts of the tissue not yet reached by the advancing bacterial front. Although the presence of G. bovis does not constitute an essential premise for DD, it seems likely that the bacterial species involved in DD vary, and that in some cases G. bovis is crucial for the development of DD lesions.
Involvement of *Guggenheimella bovis* in digital dermatitis lesions of dairy cows

Running Title: *Guggenheimella bovis* in digital dermatitis

Research paper
Pathogens and Pathogenicity

Sebastian Schlafer¹, Marcel Nordhoff², Chris Wyss³, Sarah Strub⁴, Julia Hübner¹, Dorothee Maria Gescher¹, Annett Petrich¹, Ulf B. Göbel¹ and Annette Moter¹.*

¹Institut für Mikrobiologie und Hygiene, Charité - Universitätsmedizin Berlin, Germany.
²Institut für Mikrobiologie und Tierseuchen, FU Berlin, Germany.
³Institut für Orale Biologie, Zentrum für Zahn-, Mund- und Kieferheilkunde der Universität Zürich, Switzerland.
⁴Clinic for Ruminants, Vetsuisse Faculty of the University of Berne, Bremgartenstrasse, Bern, Switzerland.

Correspondent address
*Mailing address: Charité - Universitätsmedizin Berlin, Institut für Mikrobiologie und Hygiene, Dorotheenstraße 96, D-10117 Berlin, Germany; Phone: +49 30 450524226; FAX: +49 30 450524902; e-mail: annette.moter@charite.de*
Summary

Digital dermatitis (DD) of cattle leads to lameness and a decrease of milk production and is therefore responsible for major economic losses worldwide. Although a bacterial aetiology is generally accepted, it still is unclear which microorganisms cause and/or maintain the disease. Recently, a previously undiscovered bacterial species, *Guggenheimella bovis* (OMZ 913<sup>T</sup> = CIP 108087<sup>T</sup> = DSM 15657<sup>T</sup>), has been isolated from the front of two DD lesions in Swiss cattle and suggested as a potential pathogen.

The aims of the present study were to determine the prevalence of *G. bovis* in 58 German cows affected by DD via dot blot hybridization, and to analyse the spatial distribution of *G. bovis* within the mixed bacterial flora commonly found in DD lesions by fluorescence in situ hybridization (FISH). A species-specific probe, GUBO1, was designed and evaluated. While cultured *G. bovis* was reliably identified by GUBO1, it could not be detected in any of the 58 samples submitted to PCR amplification and subsequent dot blot hybridization, nor could it be visualized in the affected tissue by FISH. Further FISH experiments were carried out on two tissue specimens of Swiss cattle tested positive for *G. bovis* by quantitative PCR and permitted to visualize the newly discovered bacteria in situ. In these biopsies *G. bovis* proved to be tissue invasive forming characteristic spherical microcolonies not only within the bacterial biofilm but also in seemingly unaffected parts of the tissue not yet reached by the advancing bacterial front. Although the presence of *G. bovis* does not constitute an essential premise for DD, it cannot be ruled out that the causative bacterial agents in different clinical cases of DD vary and that in some cases *G. bovis* might be crucial for the development of DD lesions.

Keywords: *Guggenheimella*, digital dermatitis, in situ hybridization, biofilm
Introduction

Digital dermatitis (DD) was first described by Cheli & Mortellaro in 1974 (Cheli & Mortellaro, 1974) and is an ulcerative acute or chronic inflammatory disease affecting the bovine foot. DD lesions most frequently involve the plantar skin areas proximal to the coronet of the hind limbs of dairy cattle (Blowey & Sharp, 1988; Read & Walker, 1998) and constitute an intensely painful condition, which may persist for weeks and even months impairing the general condition of the affected cattle. Episodes of lameness, weight loss and decrease of milk yield are consequences frequently described (Blowey, 1990; Hernandez et al., 2002; Laven, 2001; Laven & Logue, 2006; Murray et al., 1996). DD has been observed in various parts of the world (Brown et al., 2000; el-Ghoul & Shaheed, 2001; Enevoldsen et al., 1991; Holzhauer et al., 2006; Milinovich et al., 2004; Rodriguez-Lainz et al., 1998; Somers et al., 2003; van Amstel et al., 1995; Weaver & Court, 1993; Wells et al., 1999), its incidence increasing constantly over the past decades (Read & Walker, 1998; Somers et al., 2003; Wells et al., 1999). Up to 90% of the dairy cattle herds have been found to be affected (Laven & Logue, 2006; Read & Walker, 1998; Rodriguez-Lainz et al., 1996; Rodriguez-Lainz et al., 1998; Somers et al., 2003; Wells et al., 1999). Thus it constitutes an important economic factor and warrants intensive research. However, although a bacterial involvement is evident, the aetiology of DD is still under discussion. Treponemes but also various other eubacterial organisms have been isolated from DD lesions and have been supposed as potential pathogens (Blowey et al., 1994; Choi et al., 1997; Collighan & Woodward, 1997; Demirkan et al., 1998; Grund et al., 1995; McLennan & McKenzie, 1996; Moter et al., 1998; Walker et al., 1995).

Recently, high numbers of a previously undiscovered bacterial species, *Guggenheimella bovis*, have been found in two independent cases of DD (Simmental x Red Holstein heifers) in Switzerland. The obligate anaerobic short to coccoid Gram-positive rods have been isolated from the very front of both lesions and display a chymotrypsin-like proteolytic activity (Wyss et al., 2005). Both findings suggest an important role of *G. bovis* in the aetiology of DD. In the present study dot blot hybridization experiments were performed to determine the prevalence of *G. bovis* in German dairy cows suffering from DD. Furthermore, fluorescence in situ hybridization (FISH) was used to analyse the distribution of *G. bovis* within DD lesions and its topographical relation to other potential pathogens.
Methods

Processing of tissue specimens for dot blot hybridization. Biopsies (0.7 cm in diameter) were taken from typical DD lesions of 58 affected dairy cows (Holstein Friesian breed (n = 49), Red Holstein breed (n = 5), Fleckvieh (n = 4)) from different farms in Germany and transported to the laboratory immediately. DNA isolation, subsequent PCR amplification and preparation of dot blot membranes were performed as described previously (Choi et al., 1997; Moter et al., 2006).

Processing of tissue specimens for FISH. Parts of the tissue of each of the 58 biopsies were fixed and embedded in cold polymerizing resin (Technovit 8100, Kulzer, Wehrheim, Germany) as previously reported (Moter et al., 1998). The blocks were sectioned on a rotary microtome (Type DDM 0036, Medim, Baar, Switzerland) using steel knives with hard metal edges. Tissue sections (3-5 µm) were straightened on sterile water, placed on silanized slides (Starfrost, Burgdorf, Germany) and stored at 4 ºC. Following the evaluation of these samples, two additional biopsies from a study on Swiss cattle (Strub et al., 2007) were included in the experiments and processed in the same way.

Oligonucleotide probes. Probe EUB 338 (Amann et al., 1990), which is complementary to a region of the 16S rRNA gene conserved in the domain Bacteria, was used in dot blot hybridization as positive control to check successful PCR amplification and in FISH to visualize the entire bacterial population in the specimens. The species-specific probe GUBO1 (5'-CCAGTGGCTATCCCTGTGTGAAGG-3’), corresponding to position 135-158 in Escherichia coli 16S rRNA, was designed after comparative sequence analysis of close phylogenetic neighbours to G. bovis. To assess specificity, the probe sequence was compared to all 16S rRNA entries at the EMBL and GenBank databases (as of February 2007), making use of the Husar program package (DKFZ, Heidelberg, Germany), and to the sequences deposited in the Ribosomal Database Project II (Maidak et al., 2001). The probe was checked for its practical use in hybridization experiments by using the program OLIGO (version 4.0).

Bacterial Strains. To optimize the dot blot hybridization and FISH conditions, G. bovis (OMZ 913T = CIP 108087T = DSM 15657T) was used as positive and Tindallia magadiensis (DSM 10318T), being the closest cultured relative, as negative control (Wyss et al., 2005).
**Dot blot hybridization.** Dot blot hybridization experiments to detect *G. bovis* were performed as described previously (Choi et al., 1997; Moter et al., 2006). PCR-amplified products gained from fixed cells of *G. bovis* and its closest cultured relative *T. magadiensis* were included in all experiments as positive and negative controls respectively. All hybridizations with the probes EUB 338 (Amann et al., 1990) and GUBO1, both synthesized commercially (biomers.net, Ulm, Germany), were performed at a temperature of 54 °C, while stringency washes were carried out at 60 °C with a washing buffer containing 2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) - 0.1% SDS for EUB 338 and 5x SSC - 0.2% SDS for GUBO1. After detection of the digoxigenin-labelled probes, Xwi-ray films were exposed to the membranes for 1 to 48 hours. After stripping as reported previously (Moter et al., 2006), identical membranes were re-used for further hybridization experiments.

**FISH.** The probe EUB 338 was 5' end-labelled with fluorochrome Cy5 (indodicarbocyanine) and GUBO1 was 5' end-labelled with fluorochrome Cy3 (indocarbocyanine). Both probes were applied simultaneously. FISH experiments were performed as described previously (Sunde et al., 2003), except for mounting the slides with Vectashield containing DAPI (4,6-diamidino-2-phenylindoldihydrochlorid) (Vector Laboratories, Orton Southgate, UK). Hybridizations were carried out at a temperature of 50 °C for 2 to 3 hours. In all experiments fixed cells of *G. bovis* and *T. magadiensis* served as positive or negative controls respectively. To adjust the stringency of GUBO1, FISH experiments were performed incubating fixed cells of *G. bovis* and *T. magadiensis* using different hybridization mixes with formamide concentrations rising in steps of 5% (v/v) from 0% (v/v) to 75% (v/v). Several pictures with a fixed exposure time were taken of each bacterial species at each level of formamide. The program daime (digital image analysis in microbial ecology) (Daims et al., 2006) was used to measure the signal intensity of the stained bacteria at different concentrations of formamide. While the signal intensity of *T. magadiensis* due to unspecific binding of the probe decreased largely at formamide concentrations of 10% and above, the intensity of *G. bovis* remained stable up to formamide concentrations of 30% (v/v) (data not shown). Thus, FISH of the tissue sections was carried out with hybridization buffer containing 30% (v/v) of formamide.

**Epifluorescent microscopy.** To view the bacteria in sections processed for FISH an epifluorescence microscope (AxioPlan II, Zeiss, Jena, Germany) equipped with a 100 W high
pressure mercury lamp (HBO 103, Osram, Munich, Germany) and 10x, 40x and 100x objectives was used. Narrow band filter sets HQ F31-000, HQ F41-007 and HQ F41-008 (AHF Analysentechnik, Tübingen, Germany) were used to analyse the DAPI, Cy3 and Cy5 signals respectively. Digital images were generated with an AxioCam HRC (Zeiss) making use of the AxioVision 4.4 software.

Results and Discussion

When carried out with the probe EUB 338, dot blot hybridization experiments indicated the presence of bacteria in all of the 58 samples as well as in the positive and negative controls and thus confirmed successful PCR amplification. Using the specific probe GUBO1 under stringent hybridization conditions, *G. bovis* could not be detected in any of the clinical samples while only the positive control yielded a strong signal (Fig. 1). In all FISH experiments performed as determined above cultured *G. bovis* was reliably detected both by the specific probe GUBO1 and by the eubacterial probe EUB 338, while *T. magadiensis* could only be detected by the probe EUB 338 (Fig. 2). All of the examined DD samples from German cattle showed large amounts of various morphological types of bacteria stained by EUB 338 and displayed the characteristic structure of DD ulcers (Fig. 3) with spirochetes or fusiform bacteria being the very outriders invading the tissue at the front-of-lesion (Nordhoff & Wieler, 2005) (Fig. 4). However, *G. bovis* could not be visualized in any of these tissue slides, neither in the superficial parts of the ulcers nor in the centres of the lesions and in particular not at the apical borders (Fig. 3).

While the findings of Wyss et al. (Wyss et al., 2005) strongly suggest an aetiological role of *G. bovis* in the two examined cases of DD, the bacteria being isolated from the very front of the lesions and displaying a proteolytic activity, it is unlikely that their involvement is constitutional for the formation of DD ulcers in cattle. In none of the 58 examined tissue samples *G. bovis* could be found. It is thus not present in the lesions at all or else only present in numbers below the detection limits of FISH and dot blot hybridization. Even in the latter case it remains questionable if such minute amounts of a bacterial species are likely to influence the pathogenetic process of DD in a significant way.

While this work was in progress, another study on the prevalence of *G. bovis* in DD lesions was conducted (Strub et al., 2007). Strub et al. examined tissue samples of 20 affected cows
from Swiss farms by quantitative PCR and detected *G. bovis* in four out of 20 animals, concluding that an involvement of this organism in the aetiology of DD is improbable considering the low prevalence – a conclusion which is consistent with the results of our epidemiology on German cattle. Nevertheless, we submitted two of the Swiss biopsies tested positive for *G. bovis* to FISH to determine its role in the architecture of DD biofilms. We succeeded in visualizing *G. bovis* in these tissue sections in high numbers. Only few of these bacteria appeared as single cells, while most of them formed characteristic spherical microcolonies. Some of these colonies were observed among the other bacteria in clearly affected areas of the biopsy (Fig. 5), but the majority of them could be found in deeper, seemingly unaffected parts of the tissue. The biofilm structure of the *Guggenheimella*-positive ulcers and the bacterial morphotypes involved differed considerably from the characteristic, spirochaete-dominated lesions we observed in the 58 biopsies of German cows.

These results underline that FISH is a valid tool offering detailed information about the tissue distribution of one or more bacterial species in DD biofilms. They prove that previous detection of *Guggenheimella* (Strub et al., 2007; Wyss et al., 2005) has not been due to contamination by environmental bacteria. *G. bovis* can be part of the bacterial population in DD lesions and it is tissue invasive. As the organism could even be visualized in unaffected parts of the biopsy way ahead of the advancing bacterial front, one can conjecture that its role for the development of the DD biofilm might be an important one, that, in certain cases, it might prepare the ground for the following bacterial invasion. However, in the vast majority of the examined biopsies *G. bovis* could not be detected at all, and it is thus highly improbable that its presence constitutes an essential prerequisite for the disease. Considering the striking morphological differences between these Swiss lesions on the one hand and the 58 German lesions on the other, it is tempting to speculate whether there is more than just one entity of DD, that the process of mixed bacterial infection and inflammation leading to the ulcers is not always alike, and that in one of at least two entities the participation of *G. bovis* might be decisive. However, further and more comprehensive epidemiological data about the various potential DD pathogens, *Guggenheimella bovis* among them, need to be gained. One cannot overestimate the importance of in situ techniques for this purpose.
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References


**Figure Legends**

**Fig. 1.** Dot blot hybridizations of the identical membrane using the eubacterial probe EUB 338 (a) and the species-specific probe GUBO1 (b). In lanes A to E and fields F1 to F3 PCR-amplified products from DD lesions of 58 affected German dairy cows were applied. In fields F10 and F11 PCR products from *G. bovis* (F10) and its closest cultured relative, *T. magadiensis* (F11) were applied as positive and negative controls respectively. Fields F4 to F9 were left empty. (a) Strong signals in all fields prove successful PCR amplification. (b) *G. bovis* was not detected in any of the clinical samples, while the positive control yielded a strong signal.

**Fig. 2.** Simultaneous hybridization of fixed cells of *G. bovis* (a and c) and *T. magadiensis* (b and d) with the probes EUB 338-Cy5 (magenta) and GUBO1-Cy3 (bright orange). (a and c) Identical microscopic fields show detection of *G. bovis* by both EUB 338 (a) and GUBO1 (b) whereas detection of *T. magadiensis* by EUB 338 only (b) and not GUBO1 (d) proves specificity of the FISH experiment.

**Fig. 3.** FISH on a tissue section of a typical DD lesion using probes EUB 338-Cy5 and GUBO1 and unspecific nucleic acid stain DAPI. (a and b) Overview. (a) Overlay of the Cy5- and FITC-filter sets shows the bacterial biofilm (magenta) while background fluorescence (green) allows orientation within the tissue. (b) Same microscopic field using the Cy3-filter set. (c to f) Higher magnifications of the inserts. (c) Overlay of the Cy5- FITC- and DAPI filter sets shows massive bacterial invasion (magenta), autofluorescent erythrocytes (green) and host cell nuclei (blue) in the superficial part of the ulcer. (d) No *G. bovis* is seen in the same microscopic field using the Cy3- and DAPI- filter sets. (e and f) Likewise, *G. bovis* was not detected in the central part of the biofilm.

**Fig. 4.** FISH of the apical border of the ulcer (as indicated in Fig. 3a) using EUB 338-Cy3 and DAPI. Overlay of Cy3-, FITC- and DAPI-filter sets shows bacteria (orange) and cell nuclei (blue) in the deepest part of the biofilm. Single spirochetes (arrowheads) invade the tissue at the front of lesion.

**Fig. 5.** FISH on a tissue section of a DD biopsy tested positive for *G. bovis* by PCR. Simultaneous hybridization with the probes EUB 338-Cy5 and GUBO1-Cy3 combined with DAPI stain. (a-c) Overlay with the Cy3-, Cy5-, FITC- and DAPI filter-set. (a) Already in the overview distinct round colonies of *G. bovis* (orange) are visible besides the massive bacterial biofilm (magenta). (b) High resolution of the insert shows two microcolonies of *G. bovis* visualized by GUBO1 (orange) next to the bacterial biofilm (magenta). Note that the bacterial
morphotypes involved in this lesion differ considerably from those in Fig. 3 and Fig. 4. (c) A solitary microcolony of G. bovis detected by GUBO1 at the apical border of the ulcer.

The online version of this paper contains two supplementary movie files.

**Movie 1.** Typical DD lesion with spirochetes and fusiform bacteria invading the tissue. Deconvolution of a Z-stack reveals the spiral morphotype of the bacterial outriders (orange) detected by the eubacterial probe EUB 338-Cy3.

**Movie 2.** FISH of a *Guggenheimella*-positive DD ulcer. Z-stacking through the section shows the spherical shape of the *G. bovis* microcolony detected by GUBO1-Cy3 (orange). Note the considerable morphological differences of the bacteria visualized by EUB 338-Cy5 (magenta) as compared to movie 1 and the absence of spirochetes.