Potential bacterial core species associated with digital dermatitis in cattle herds identified by molecular profiling of interdigital skin samples

Martin W. Nielsen, Mikael L. Strube, Anastasia Isbrand, Worood D.H.M. Al-Medrasi, Mette Boye, Tim K. Jensen, Kirstine Klitgaard*

National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark

ABSTRACT

Although treponemes are consistently identified in tissue from bovine digital dermatitis (DD) lesions, the definitive etiology of this debilitating polymicrobial disease is still unresolved. To study the microbiomes of 27 DD-infected and 10 healthy interdigital skin samples, we used a combination of different molecular methods. Deep sequencing of the 16S rRNA gene variable regions V1–V2 showed that *Treponema, Mycoplasma, Fusobacterium* and *Porphyromonas* were the genera best differentiating the DD samples from the controls. Additional deep sequencing analysis of the most abundant genus, *Treponema,* targeting another variable region of the 16S rRNA gene, V3–V4, identified 15 different phylotypes, among which *Treponema phagedenis*-like and *Treponema refringens*-like species were the most abundant. Although the presence of *Treponema* spp., *Fusobacterium necrophorum* and *Porphyromonas levii* was confirmed by fluorescence *in situ* hybridization (FISH), the results for *Mycoplasma* spp. were inconclusive. Extensive treponemal epidermal infiltration, constituting more than 90% of the total bacterial population, was observed in 24 of the 27 DD samples. *F. necrophorum* and *P. levii* were superficially located in the epidermal lesions and were present in only a subset of samples. RT-qPCR analysis showed that treponemes were also actively expressing a panel of virulence factors at the site of infection. Our results further support the hypothesis that species belonging to the genus *Treponema* are major pathogens of DD and also provide sufficient clues to motivate additional research into the role of *M. fermentans, F. necrophorum* and *P. levii* in the etiology of DD.

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1. Introduction

Bovine digital dermatitis (DD) is presently the most common cause of lesions associated with lameness in dairy cattle worldwide (Laven and Logue, 2006). This debilitating disease has a huge impact on animal welfare and productivity (Losinger, 2006). DD most probably has a bacterial etiology because no virus or fungus of any significance has been identified from the lesions (Krull et al., 2014). Likewise, the immunological response of afflicted cows and the positive effect of antibiotic treatment seem to support this notion (Demirkan et al., 1999). Various species of spirochetes have been cultivated from the affected lesions (Pringle et al., 2008; Evans et al., 2009); and with the advent of 16S rRNA gene sequencing and fluorescence *in situ* hybridization (FISH), it has been shown that a considerable number of *Treponema*

phylotypes are present in DD lesions (Moter et al., 1998; Klitgaard et al., 2008; Rasmussen et al., 2012). Phylogenetically, the identified treponemes mainly belong to 4 major groups, *Treponema phagedenis*-like, *Treponema denticola/Treponema pedis*-like, *Treponema medium/Treponema vincentii*-like and *Treponema refringens*-like phylotypes (Yano et al., 2010; Santos et al., 2012; Klitgaard et al., 2013). These bacteria are consistently reported to be located deep within the affected tissue (in the front part of the lesion) and to outnumber other bacterial morphotypes. Furthermore, their presence is always associated with degenerative and necrotic changes of the infected tissue (Choi et al., 1997). These tissue-destructive properties have been reported to be enhanced by and to probably be dependent upon the presence of other micro-organisms found in these polymicrobial infections (Edwards et al., 2003). However, it must be emphasized that Koch's postulates have not been fulfilled for DD, and the definitive etiology of the disease is therefore still unresolved.

In addition to treponemes, the range of taxa identified from DD lesions by bacteriological, histopathological, and molecular biological investigations include the genera *Porphyromonas,Prevotella*, *Fusobacterium*, *Campylobacter*, *Bacteroides*, *Mycobacterium*, *Mycoplasma* and *Guggenheimella* (Wyss et al., 2005; Santos et al., 2012; Krull et al., 2014). Many of these bacterial groups, however, seem to be primarily located in the superficial parts of the lesions, and their potential roles in the pathogenesis of DD are still unresolved. The bacterium *Dichelobacter nodosus*, the cause of bovine foot rot, is commonly seen invading the epidermis in DD lesions (Rasmussen et al., 2012).

The aim of the present study was to determine whether other bacteria, besides treponemes, are consistently associated with DD and to investigate whether we could link distinct phylotypes of *Treponema* to specific stages of DD lesion development as previously indicated (Krull et al., 2014; Zinicola et al., 2015). FISH analysis was applied to visualize and localize selected microbial taxa identified by deep sequencing of the 16S rRNA gene. Finally, the expression of a number of known or putative *Treponema* virulence genes in DD lesions was measured by reverse transcriptase real-time PCR (RT-qPCR). To date, only the immunological host gene response has been investigated for DD (Zuerner et al., 2007), and the active expression of candidate virulence genes would be a further indication that *Treponema* are actively involved in the etiology of this disease.

2. Materials and methods

2.1. Sample collection and preparation

All sample material was collected from a Danish cattle slaughterhouse. Tissue samples were collected from the hind feet of slaughtered Holstein-Friesian dairy cattle after routine slaughter procedures. The feet were gently rinsed with water and cleaned with a soft-haired brush to remove any gross organic debris, and the dorsal/posterior skin between the digits was classified as early (M1), acute ulcerative (M2), healing (M3) or chronic lesions (M4) according to Döpfer et al. (1997).

Tissue samples were collected with a sterile scalpel and transferred to a sterile Petri dish. The samples were subdivided and stored for histologic examination and DNA purification. The sample portions intended for 16S rRNA gene analysis were immediately transferred to a nucleic acid stabilization solution (RNA*later*®, Ambion, Austin TX) and stored at −20 °C. Samples intended for histopathology analysis were fixed in 10% neutral buffered formalin solution, dehydrated, embedded in paraffin wax, sectioned (5-μm thick) and mounted on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany).

2.2. Histopathology

All tissue samples were stained with hematoxylin and eosin (H&E) for histopathological evaluation. The degree of epidermal damage and the inflammatory response of the dermis were scored from 0 to 2 according to Rasmussen et al. (2012), with minor modifications. A score of 0 was defined as normal epidermis or mild epidermal damage with some epithelial proliferation and hyperkeratosis, dermis being unaffected. Score 1 was defined as epithelial proliferation and acanthosis (with ballooning degeneration and mal-keratinization) while the inflammatory response was seen as mild to moderate increase in the number of lymphocytes and mononuclear cells. Score 2 was defined as severe epithelial proliferation, acanthosis and exudation, erosion or necrosis of the dermal papilla and moderate to severe infiltration with lymphocytes and/or mononuclear cells in dermis and perivascular dermatitis.

2.3. Fluorescent in situ hybridization (FISH)

The oligonucleotide probes used in this study are listed in Table S1 and include probes specific for Domain Bacterium, *Treponema* spp., *Fusobacterium necrophorum*, *Dichelobacter nodosus*, *Porphyromonas levii*, *Mycoplasma* spp., and *Mycoplasma fermentas*. Newly designed oligonucleotide probes were selected using the software ARB (http://www.arb-home.de). The oligonucleotide probes were 5′ labeled with fluorescein isothiocyanate (FITC) or the isothiocyanate derivative Cy3 (Eurofins MWG Operon, Ebersberg, Germany). The hybridization was carried out at 45 °C with 100 μl of hybridization buffer (10 μl of 1 M Tris [pH 7.2], 18 μl of 5 M NaCl, 1 μl of 10% sodium dodecyl sulfate, 71 μl of H_2O) and 500 ng of each probe for 16 h in a Sequenza slide rack (Thermo Shandon, Cheshire, United Kingdom). The sections were then washed three times in prewarmed (45 °C) hybridization buffer for 9 min and subsequently washed three times in prewarmed (45 °C) washing solution (10 μl of 1 M Tris [pH 7.2], 18 μl of 5 M NaCl, 72 μl of H_2O). The sections were rinsed in water, air dried, and mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA) for epifluorescence microscopy. An Axioimager M1 epifluorescence microscope equipped for epifluorescence with a 100-W HBO lamp and filter sets 43 and 38 was used to visualize Cy3 and FITC, respectively. Images were obtained using an AxioCam MRm version 3 FireWiremonocrome camera and AxioVision software, version 4.5 (Carl Zeiss, Oberkochen, Germany).

2.4. Nucleotide extraction

DNA and RNA were extracted from the biopsies using an AllPrep® DNA/RNA/miRNA kit (Qiagen, Hilden, Germany). Portions (20 mg) of stabilized tissue were first homogenized in 600 μl of RTL plus buffer (included in the kit). A sterile 5-mm steel bead (Qiagen) was added, and samples were run two times on a TissueLyser II (Qiagen) at 20 Hz for 2–5 min per run. All the subsequent procedures were performed according to the protocols of the supplier. After RNA extraction, the material was additionally treated with TURBO™ DNase, according to the protocol provided by the manufacturer (Ambion), to ensure total DNA degradation in the RNA preparations. For the evaluation of DNA and RNA concentration and purity, samples were analyzed on a NanoDrop ND-1000 spectrophotometer (Fisher Scientific, Wilmington, MA). DNA samples with $A260/A280$ ratios >1.5 were selected for further analysis. The RNA quality was estimated on an Agilent Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA).

2.5. Reverse transcription

Reverse transcription was performed using a QuantiTect Reverse Transcription kit according to the manufacturers instructions (Qiagen). The concentration and quality of cDNA samples were measured with a NanoDrop ND-1000 spectrophotometer (Fisher Scientific). All cDNA samples with $A260/A280$ ratios ≥1.8 were included in the downstream analysis.

2.6. Preparation of 16S rRNA gene amplicon libraries and sequencing

PCR amplification of DNA was accomplished with a universal bacterial primer set (Wilmotte et al., 1993) and a *Treponema*-specific primer set (Klitgaard et al., 2008) targeting the V1–V2 region and the V3–V4 hyper variable regions of the 16S rRNA gene (primer sequences listed in S1). Each sample was amplified with unique forward and reverse primers that included an added hexamer barcode at their 5′ ends. Amplification PCRs were performed in 50-μl reaction mixtures containing 5 μl of 10xPCR Gold Buffer (Applied Biosystems, Foster City, CA, USA) 1.5 mM