Quantitation of *Guggenheimella bovis* and treponemes in bovine tissues related to digital dermatitis

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Abstract

Digital dermatitis is an inflammation of uncertain aetiology in the skin of the foot of cattle. In 2005, a novel microorganism, *Guggenheimella bovis*, was isolated from the advancing front of digital dermatitis lesions, suggesting a possible role in pathogenesis. In the present study, tissue samples of 20 affected cows were examined by quantitative PCR for *G. bovis*, treponemes and the total eubacterial load. High numbers of eubacteria and treponemes were found in most lesions, whereas only a few lesions contained *Guggenheimella*, and only at low concentrations. The results argue against the relevance of *G. bovis* in the aetiology of digital dermatitis in cattle, but are consistent with a role for treponemes.

Introduction

Digital dermatitis is an inflammation of the skin at the heel and bulbs of the feet of cattle. The disease, first described in 1974, is also called papillomatous digital dermatitis, hairy foot warts, Mortellaro-disease or strawberry foot (Cheli & Mortellaro, 1974). More rarely, lesions have been also found in the foot of the forelimb, in the interdigital skin and in the area of the coronary band. The early stage of the lesion shows a circumscribed granulomatous area with hairs standing erect, covered with exudates. Typically, ulceration with granulation tissue is present, which is painful upon palpation. Proliferative, hyperkeratotic lesions (papillomatous, hairy foot warts) are seen as an aggressive, chronic form.

Digital dermatitis is an important herd health problem in cattle worldwide causing great economic loss (Yeruham *et al.*, 2000; Losinger, 2006). The disease is common in an increasing number of countries including Switzerland (Luginbühl & Kollbrunner, 2000). It causes local pain, lameness and a decrease in milk production, primarily because of reduced feed intake. Risk factors include poor conditions of hygiene, contact with slurry, humidity and free stall housing (Wells *et al.*, 1999).

An infectious aetiology is suggested because of the fast spread in the herd and the responsiveness to antibiotics

(Read & Walker, 1998). However, until now, a causative agent of digital dermatitis has not been identified. Viruses have not been isolated or detected from affected tissue (Bassett *et al.*, 1990). Several bacteria have been found, among others *Campylobacter faecalis*, *Dichelobacter nodosus*, *Bacteroides* spp. and *Fusobacterium necrophorum*. Spirochetes from the genus *Treponema* have attracted the most attention. In 1995, Walker *et al.* isolated two groups of spirochetes from affected dairy cows that were phenotypically most consistent with the genus *Treponema* (Walker *et al.*, 1995). Döpfer *et al.* (1997) showed the presence of spirochetes by microscopy of digital dermatitis lesions. In 1999, Demirkan *et al.* succeeded in isolating and cultivating *Treponema* from lesions (Demirkan *et al.*, 1999a). Several studies used molecular methods to confirm the presence of treponemes: in 1997, Choi *et al.* identified five treponemal phylotypes from digital dermatitis lesions by PCR and demonstrated the presence of *Treponema denticola*-like spirochetes in suspensions from digital dermatitis lesions by FISH. Rijpkema *et al.* (1997) amplified spirochetal DNA by PCR in 1997 and assigned them next to *Treponema denticola*. A novel *Treponema* species was isolated from lesions and described as *Treponema brennaborense* (Schrank *et al.*, 1999). Serological examinations also indicated an

association between spirochetes and digital dermatitis (Walker *et al.*, 1997; Demirkan *et al.*, 1999b; Murray *et al.*, 2002; Dhawi *et al.*, 2005).

In a recent study of two cases of digital dermatitis in Switzerland, only one of the lesions contained microscopically detectable treponemes. From the front of both lesions, gram-positive, anaerobic rods described as *Guggenheimella bovis* were isolated (Wyss *et al.*, 2005). The presence of these potentially proteolytic organisms at the advancing front of the lesions suggested an aetiological role. The objective of this study was to collect epidemiological data on the presence of *G. bovis* and treponemes in digital dermatitis lesions in Switzerland and to evaluate their role in the aetiology of this disease.

Material and methods

Study design and collection of clinical material

Twenty cattle with clinical signs of digital dermatitis but without antibiotic pretreatment and tested free from bovine virus diarrhoea (BVD) antigen were selected for this study. The age of the cattle ranged from 26 to 132 months, with a mean age of 58 months. The breeds Holstein Friesian (11), Red Holstein and Red Holstein×Simmental crossbreeds (4) and Swiss Braunvieh (5) were represented.

One affected animal and one macroscopically healthy control cow per farm were selected for sampling. Preparation of the foot included clipping, cleaning of the area of the bulbs, interdigital anaesthesia with 20 mL of Lidocain 2%, followed by final disinfection with alcohol and diluted PVP-iodine solution. Using sterile instruments, a superficial sample ('surface', thickness of about 5 mm) of the lesion, followed by a 6 mm diameter biopsy (biopsy punch, Stiefel Laboratorium GmbH, Offenbach am Main, Germany) from the advancing front of the lesion ('front') were collected from affected animals. From a healthy foot of each affected animal and from a hind foot of a control animal of the corresponding herd, a deep skin scab ('scab') was additionally sampled. On five farms, control skin scabs were not available, because all animals were macroscopically affected by digital dermatitis. Twenty control 'surface' and 'front' samples each were collected at the slaughterhouse from feet of cattle free from digital dermatitis.

Biopsy material for quantitative PCR was kept in sterile tubes on ice during transportation to the lab and stored at $-20\,^{\circ}$C until use.

DNA preparation

The FastDNA$^{\circledR}$ SPIN kit (Qbiogene, Inc., CA) was used according to the standard protocol of the manufacturer. For DNA isolation of tissue, 100–150 mg of the biopsies 'surface' and 'front' were minced with a scalpel. Skin scab was used as sampled. The tissue samples were run twice in the FastPrep$^{\circledR}$ instrument (Qbiogene) to improve the lysis. For use as amplification control, DNA was extracted from pure bacterial cultures, grown as described by Wyss *et al.* (2005).

Quantitative PCR (QPCR)

Quantitative PCR with minor-groove binder Taqman probes was used to measure the load of 'total eubacteria', *G. bovis* and treponemes. PRIMER EXPRESS Software (Applied Biosystems, CA) and ARB phylogenetic software (Ludwig *et al.*, 2004) were used to design primers and probe specific for 16S rRNA genes of *G. bovis* and treponemes (see Table 1).

The specificity of the *G. bovis* assay was tested with DNA isolated from *Eubacterium nodatum* ATCC 33099[T], *Treponema denticola* ATCC 35405[T], *Treponema lecithinolyticum* OMZ 684[T], *Treponema 'vincentii'* OMZ 800, *Guggenheimella* strain OMZ 915, *Treponema medium* ATCC 700293[T], *Tindallia magadiensis* OMZ 951[T] and *Treponema brennaborense* DSMZ 12168[T]. They showed no amplification, except *Guggenheimella* strain OMZ 915, whose 16S rRNA gene sequence is 100% identical to that of strain OMZ 913.

Table 1. Primers and probes used in the quantitative PCR

Specificity	Name	Function	Sequence 5′–3′	Length of amplicon (bp)
G. bovis	Gbov-574F	Forward	GTGAAAGGCAAGGGCTTAACC	63
	Gbov-627R	Reverse	CCCCTCCTGCACTCAAGCTA	
	Gbov-606T	Probe	6-FAM-TTGTTAGCCATTGAAACCA-MGB	
Treponemes	Tre-1F	Forward 1	AAGGCAACGATGGGTATCC	128
	Tre-2F	Forward 2	AAGGCGACGATGGGTATCC	
	Tre-3F	Forward 3	AAGGCGATGATGGGTATCC	
	Tre-R	Reverse	GCGTCGCTCCGTCAGACT	
	Tre-T	Probe	VIC-GACACATTGGGACTGAGATA-MGB	
Universal	Uni-F	Forward	TCCTACGGGAGGCAGCAGT	466
	Uni-R	Reverse	GGACTACCAGGGTATCTAATCCTGTT	
	Uni-T	Probe	6-FAM-CCAGCAGCCGCGGTA-MGB	

For detection of treponemes, an assay including three different forward primers was chosen to cover as many *Treponema* species as possible. This PCR assay was tested with *Treponema denticola* ATCC 35405[T], *Treponema lecithinolyticum* OMZ 684[T], *Treponema medium* ATCC 700293[T], *Treponema brennaborense* DSMZ 12168[T] (Schrank *et al.*, 1999) and *Treponema 'vincentii'* OMZ 800. All of the tested *Treponema*-DNA yielded amplicons. In addition, according to ARB, at least one of the forward primers, the reverse primer and the probe matched 100% with sequences from *Treponema denticola*, *Treponema putidum*, *Treponema lecithinolyticum*, *Treponema brennaborense*, *Treponema phagedenis*, *Treponema vincentii*, *Treponema medium*, *Treponema maltophilum* and several uncultured *Treponema* sp. These organisms should therefore be detectable with this assay.

For quantitation of the eubacterial load, a recently described assay was used (Nadkarni *et al.*, 2002). But due to the presence of a minor-groove binder, the length of the universal probe was shortened from 23 bases to 15 bases. This shorter probe length should lead to a broader coverage of bacteria. The universal assay was tested positive with the strains *Treponema denticola* ATCC 35405[T], *Treponema lecithinolyticum* OMZ 684[T], *Treponema medium* ATCC 700293[T], *Treponema brennaborense* DSMZ 12168[T], *Treponema 'vincentii'* OMZ 800, *Eubacterium nodatum* ATCC 33099[T], *Eubacterium saphenum* OMZ 917[T], *G. bovis* OMZ 913[T] and 915 and *Tindallia magadiensis* OMZ 951[T].

Preparation of standards

Linearized plasmids harbouring parts of 16S rRNA genes were used as standards. For this, chromosomal DNA from *G. bovis* OMZ 913 and *Treponema brennaborense* OMZ 952 served as a template in a PCR reaction using primers 27F and 1492R (Lane, 1991). The PCR product was cloned into pGEM-T (Promega, WI). The resulting plasmids were isolated from *E. coli* using the Jetstar Midi[®] kit (Genomed, Löhne, Germany). The DNA concentration was determined by measuring the absorbance at 260 nm. The DNA was digested with PstI to linearize the plasmid. A 10-fold dilution series of DNA in TE buffer (1 mM Tris-HCl, 0.01 mM EDTA, pH 8.0) served as the standard.

Quantitative PCR run

Each reaction contained 12.5 μL of TaqMan[®] Universal PCR MasterMix (Applied Biosystems, CA) and 2 μL of template DNA in a total volume of 25 μL. The primer and probe concentrations were optimized for each of the assays as follows: for *G. bovis*, 300 nM of both forward and reverse primer and 200 nM for the probe, for treponemes, 900 nM of the reverse primer, 300 nM of each of the three forward primers and 200 nM of the probe and for the universal assay

300 nM of each primer and 150 nM of the probe. Each sample, isolated with the FastPrep kit, was 10-fold diluted with water to avoid inhibition. Amplification was performed in an ABI PRISM[®] 7000 Sequence Detection System (Applied Biosystems) using the following profile: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. For the universal assay, annealing and extension were at 60 °C for 2 min, which proved to be necessary because of the larger product size.

Duplicate samples were used in the quantitative PCR. For each assay and run, four negative controls were included.

The presence of inhibitory substances was tested in a quantitative PCR by addition of plasmid DNA in a known quantity to each reaction with genomic DNA of samples from 20 different cows. This QPCR run gave no evidence of impeded amplification.

Data analysis

Data were analysed by the Mann–Whitney *U* test, contingency table and Fisher's exact test using STATVIEW software (Abacus Concepts, Inc., CA). Values of $P < 0.05$ were considered to be significant.

Results and discussion

The aetiology of bovine digital dermatitis is still unresolved, but an important bacterial background contribution is not disputed. This view is based on the kind of spreading of the disease and its treatability with antibiotics, and is supported by the present study: On five out of the 20 farms with cases of digital dermatitis investigated, all animals of the herd were affected. The average farm in this study had 36 cows. To obtain a view on the potential importance of bacteria in this economically important disease, 'all' eubacteria, treponemes and *G. bovis* were quantitated at different locations in healthy and affected cattle. The three QPCR assays developed gave consistent results in all control situations. However, the weighing of quantitative results for the different assays with respect to the number of 16S rRNA gene copies is technically unresolved. The results obtained by quantitative PCR are summarized in Table 2.

The eubacterial load in the surface ($P < 0.0001$) and front ($P = 0.02$) samples, as determined by universal PCR, was significantly higher in affected than in healthy animals. Apparently, the damaged skin provides better conditions for the survival and growth of bacteria. The large amounts of eubacteria detected by PCR at the front of the lesion, i.e. after the surgical removal of the macroscopically affected skin, support the notion of digital dermatitis as a polymicrobial disease (Cruz *et al.*, 2005) (See Table 2, Fig. 1a). Consistent with this, histology of such front samples showed high numbers of bacteria accompanied by inflammation

Table 2. Mean quantity of target DNA copies per gram tissue detected in the three sample types with the universal, *Guggenheimella bovis* and treponeme assays. Also given are the numbers of positive and negative tested animals

	Affected*		Healthy†	
	Positive tested	Negative tested	Positive tested	Negative tested
	Uni‡		Uni	
Surface	1.3×10^9		2.4×10^7	
Front	6.7×10^7		1.6×10^6	
Scab§	7.3×10^9		3.5×10^9	
	Gbov¶	Gbov	Gbov	Gbov
Surface				
Mean	9.8×10^6	ND‖	ND	ND
Count	2	18	0	20
Front				
Mean	1.6×10^6	ND	ND	ND
Count	1	19	0	20
Scab				
Mean	1.3×10^8	ND	8.4×10^7	ND
Count	3	17	2	13
	Tre#	Tre	Tre	Tre
Surface				
Mean	1.5×10^9	ND	4.2×10^6	ND
Count	19	1	10	10
Front				
Mean	4.3×10^7	ND	2×10^5	ND
Count	17	3	12	8
Scab				
Mean	5.6×10^8	ND	3.4×10^8	ND
Count	19	1	13	2

*Animals affected with digital dermatitis from farms ($n = 20$).

†Healthy animals from the slaughterhouse ('surface' and 'front'; $n = 20$) or from farms ('scab'; $n = 15$).

‡Universal, eubacterial load.

§Scab samples should not be compared with the other sample types, as they are another sort of sample and were only taken from healthy feet.

¶*Guggenheimella bovis*.

‖ND, not detected (detection limit of 2×10^5 copies g^{-1} for *G. bovis* and 2×10^4 copies g^{-1} for treponemes and eubacteria).

#Treponemes.

Fig. 1. Box-Plot representations of the quantity of DNA copies. The figures compare healthy = DD− and affected = DD+ animals for three different sample types. The corresponding p-values represent the differences between affected and healthy animals within one sample type. (a) Quantity of the eubacterial load detected with universal primers and probe. (b) Quantity of treponemes.

and necrosis (not shown). In the skin scab of healthy and affected animals, there was no difference in the yield of bacteria detectable. The scab sample was taken from the control animals, which lived under the same environmental conditions and, according to the same eubacterial load, under the same infection pressure as the affected cattle. Proposed risk factors such as poor conditions of hygiene, moisture, type of housing, frequency and quality of claw trimming or feeding regimens do not seem to be the only predisposing factors. Some animals appear to have a higher individual susceptibility.
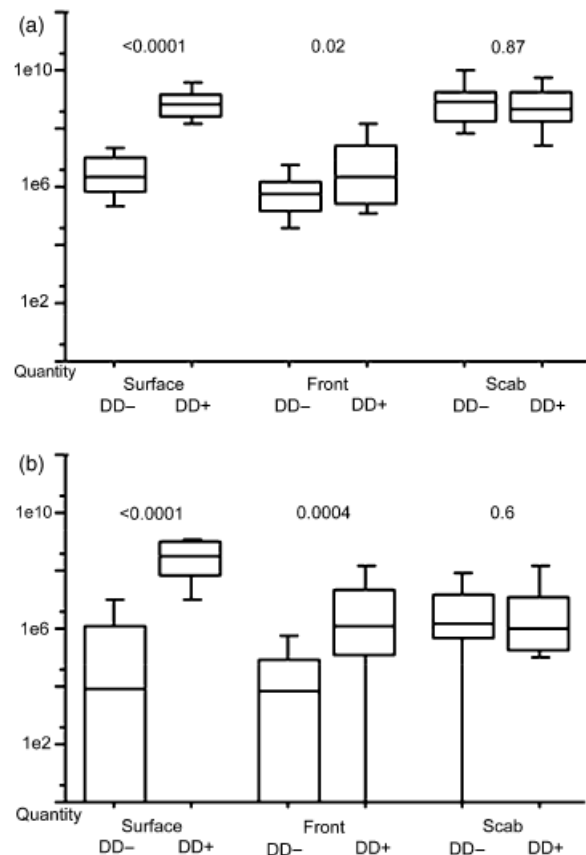
Guggenheimella bovis has only recently been discovered in two digital dermatitis lesions. In the present study, it was found in only four of 20 affected animals. No statistical differences between healthy and affected cattle were found in the number of animals that tested positive for *G. bovis* (Table 2). In healthy animals, *G. bovis* was detected only in the skin scab and there was no difference in the quantity of *G. bovis* between healthy and affected animals. Because the detection limit was 2×10^5 copies g^{-1}, it is possible that in some of the samples *G. bovis* was present in lower numbers. Nevertheless, in light of the low prevalence of *G. bovis* in affected animals it is unlikely that *Guggenheimella* is involved in the aetiology of digital dermatitis in cattle. Given the mesophilic nature of *G. bovis*, an environmental reservoir as for *Tindallia*, its closest relative, seems unlikely. This indicates an association with the host animal and/or its warm excreta. It is not clear whether the low numbers of *G. bovis* detected on the skin

represent the source of these bacteria inhabiting the lesions. The role of *G. bovis* in bovine health remains to be elucidated.

Treponemes have attracted much attention in studies of digital dermatitis in part due to their ready detection by microscopy alone. The recognition of an enormous phylogenetic diversity among isolated and even more among as yet uncultured treponemes has complicated the evaluation of their possible involvement in the disease development. In the present study, a combination of primers was used to optimally quantify treponemes as a group by PCR. As shown in Table 2, significant numbers of treponemes were detected in 19 out of 20 surface and 17 out of 20 front samples of affected animals. The healthy and affected animals showed a difference in the surface samples for treponemes with a *P*-value of 0.003. This was the only significant difference in the number of positive tested animals within sample type between healthy and affected animals. In samples from the surface ($P < 0.0001$) and the front ($P = 0.0004$) but not from the scab, the load of treponemes was significantly higher in affected as compared with control animals (Fig. 1b). This points to a close association of treponemes with the disease process of digital dermatitis. As, however, no treponemes were detectable at all in one animal with a typical lesion, the presence of treponemes does not represent a conditio sine qua non for development of digital dermatitis. Furthermore, the presence of treponemes in the front tissue of nonaffected slaughterhouse animals was surprising and needs to be taken into consideration. The presence of a higher amount of treponemes in the surface samples compared with the total bacterial load seems confusing. It may be that some *Treponema* strains were not detected by the universal PCR assay, as shown by Horz *et al.* (2005), who found that spirochetes were poorly covered by the protocol of Nadkarni *et al.* (2002). Surprisingly, 12 out of 20 front samples from healthy control animals tested positive for treponemes. Interestingly, in some of these animals, however, treponemes were not detected by PCR in the surface samples. At least in these cases, an artefact can be excluded due to carry-over from surface to front during sampling. While all *Treponema* species are motile and believed to be obligatory host associated, too little is known about ways of transmission and modes of survival outside hosts to draw definitive conclusions. It could be, that another commensal is required to cause damage. Some control animals could have suffered from digital dermatitis, recovered and the treponemes that were involved survived in the skin. It is possible that these cattle harbour a reservoir with the potential for a reinfection. Further studies including a histological search for treponemes in larger areas around the lesion are required to answer questions concerning the source and the mode of entry of the treponemes.

Acknowledgements

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