High prevalence of treponemes in bovine digital dermatitis – a molecular epidemiology

Marcel Nordhoff, Annette Moter, Kirstin Schrank, Lothar H. Wieler

Title: High prevalence of treponemes in bovine digital dermatitis – a molecular epidemiology

Authors: Marcel Nordhoff, Annette Moter, Kirstin Schrank, Lothar H. Wieler

PII: S0378-1135(08)00133-8
DOI: doi:10.1016/j.vetmic.2008.04.019
Reference: VETMIC 3996

To appear in: *VETMIC*

Received date: 7-11-2007
Revised date: 26-3-2008
Accepted date: 10-4-2008

Please cite this article as: Nordhoff, M., Moter, A., Schrank, K., Wieler, L.H., High prevalence of treponemes in bovine digital dermatitis – a molecular epidemiology, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.04.019

High prevalence of treponemes in bovine digital dermatitis – a molecular epidemiology

Running title: Detection of different *Treponema* groups in digital dermatitis

Research paper

Marcel Nordhoff[1]*, Annette Moter[2], Kirstin Schrank[1] and Lothar H. Wieler[1]

Institut für Mikrobiologie und Tierseuchen, FU Berlin[1]

Institut für Mikrobiologie und Hygiene, Charité-Universitätsmedizin Berlin[2]

Correspondent address
*Mailing address: Freie Universität Berlin, Fachbereich Veterinärmedizin
Institut für Mikrobiologie und Tierseuchen,
Philippstrasse 13, D-10115 Berlin, Germany
Phone: +49 30 20936335; Fax: +49 30 20936067;
e-mail: nordhoff.marcel@vetmed.fu-berlin.de

Keywords: *Treponema*, digital dermatitis, molecular epidemiology, FISH

Abstract

To validate the epidemiology of *Treponema* spp. associated with digital dermatitis (DD) a large number of DD samples (n = 56) were examined by DNA-DNA dot blot analyses using oligonucleotide probes specific for phylogenetic group I – VII of oral treponemes and DD-associated phylotypes DDKL-4, DDKL-12 and DDKL-20 as well as for *T. brennaborense* and *T. socranskii.* Positive hybridisation results were obtained for phylogenetic groups I, II and IV and phylotypes DDKL-4 and DDKL-12. While phylotype DDKL-4 was detected in 100 % of the samples treponemes belonging to phylogenetic group TRE I, TRE II and TRE IV were prevalent in nearly 80 % of the samples and phylotype DDKL-12 was detected in 66.1 % of the samples.

Analysis of *Treponema* groups present concurrently in the same sample revealed that a combination of TRE I - TRE II - TRE IV - DDKL-4 was most prevalent and could be detected in up to 71 % of the samples. These data indicate that this combination of different *Treponema* spp. seems to be the most important one in the pathogenesis of DD.

In contrast, *T. brennaborense* originally isolated from DD material this treponeme was not detected in any of the samples clearly indicating that this species is not absolutely associated with DD and therefore may represent only an incidental treponeme.

Fluorescence *in situ* hybridisation (FISH) obviously highlights the invasive character of DD-associated treponemes. Mainly treponemes belonging to phylogenetic group TRE I and phylotype DDKL-4 were detected in high numbers compared to the total number of bacteria and also in deeper layers of the epithelium at the transition of unaffected and affected tissue.

Our results confirm a high prevalence and diversity of *Treponema* spp. in DD lesions. In addition, our data indicate that certain combinations of *Treponema* spp. are detected much more frequently than others. Furthermore, *Treponema* spp. appear at the interface between healthy and diseased tissue underlining their importance for the pathogenesis of DD.

Introduction

Although digital dermatitis (DD) was first described by Cheli and Mortellaro nearly 30 years ago the exact aetiological cause of this disease still remains unclear (Cheli and Mortellaro, 1974; Dhawi et al., 2005).

DD is associated with unfavourable conditions in animal husbandry (Cruz et al., 2005; Rodriguez-Lainz et al., 1999; Wells et al., 1999) and several anaerobic bacterial species have been detected in DD lesions so far including *Porphyromonas* spp., *Prevotella* spp., *Fusobacterium* spp. and *Guggenheimella bovis* (Nattermann et al., 1998; Nordhoff and Wieler, 2005; Schlafer et al., 2008; Wyss et al., 2005).

In addition *Treponema* (*T.*) spp. are thought to play an important role in the pathogenesis of DD (Dhawi et al., 2005; Grund et al., 1995; Logue et al., 2005). In particular FISH analyses of DD revealed that treponemes are of prime importance in the pathogenesis of DD showing high numbers of treponemes deep within the tissue (Moter et al., 1998b). Several virulence factors have been described so far for bovine and ovine isolates (Edwards et al., 2003a) and recent cell culture assays indicate that bovine *T. phagedenis*-like spirochetes impair the innate immune system as well as wound repair functions (Zuerner et al., 2007).

Culture independent 16S rRNA gene analyses of DD samples revealed five different *Treponema*-phylotypes (Choi et al., 1997). While phylotypes DDKL-3 and DDKL-13 are closely related to *T. denticola and T. vincentii,* respectively, phylotypes DDKL-12 and DDKL-20 have no known close relatives of oral treponemes. Phylotype DDKL-4 is nearly identical to *T. phagedenis*, a non-pathogenic treponeme in humans originally isolated from the urogenital tract.

Furthermore, several bovine *Treponema* isolates assigned to the *T. phagedenis*-like, *T. denticola*-like and *T. vincentii/T. medium*-like group have been cultivated from DD lesions repeatedly while *T. brennaborense* was isolated only once (Demirkan et al., 1999; Schrank et al., 1999; Stamm et al., 2002; Walker et al., 1995).

To examine the prevalence and diversity of different *Treponema* spp. and their role in the pathogenesis of DD a large number of acute DD lesions (n = 56) from affected cows were screened for treponemes by DNA-DNA dot blot hybridisation and FISH analyses.

Material and methods

Collection of DD biopsy samples

Biopsies were taken from cows suffering from acute digital dermatitis. A total of 56 biopsies were collected from typical DD-lesions as previously described (Schlafer et al., 2008). In brief, samples were taken from typical DD lesions of 56 affected dairy cows (Holstein Friesian breed (n = 47), Red Holstein breed (n = 5), Fleckvieh (n = 4)) originating from different farms in Saxony, Brandenburg, Berlin and Bavaria, and transported to the laboratory immediately.

Before sample collection DD lesions were washed softly with water to remove dirt attached to the skin's surface.

DNA extraction from biopsies

Biopsies were cut in to small pieces and put directly into lysis buffer (500 mM Tris-HCl, pH 9.0, 20 mM EDTA, 10 mM NaCl, 1% sodium dodecylsulfate [SDS]) and stored at -20°C. For subsequent DNA extraction samples were thawed and proteinase K was added in a final concentration of 500 µg / ml. Incubation was performed at 56°C until lysis was completed. DNA was purified by a standard phenol-chloroform extraction. After precipitation with ethanol DNA was dissolved in TE buffer (10mM Tris-HCL, 1 mM EDTA, pH 7.4) and stored at -20°C.

PCR amplification of 16S rRNA gene

For PCR amplification of approximately 550 bp of 16S rRNA gene the spirochete-specific forward primer SPU1 (5´- GTYTTAAGCATGCAAGTCG-3´, position 46-64 in E. coli 16S rRNA gene) and the universal reverse primer RTU-3 (5´-GWATTACCGCGGCKGCTG-3´, position 518-535 in E. coli 16S rRNA gene) were used. Amplification of the partial 16S rRNA gene was conducted in a final volume of 50 µl containing 2,5 mM $MgCl_2$, 0,2 mM dNTP´s, 25 pmol of each primer and 2,5 U *Taq* DNA polymerase (Invitrogen, Germany). Samples were preheated at 94 °C for 5 min followed by amplification with 94 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s. A total of 30 cycles was carried out with a final elongation step at 72 °C for 10 min. Successful amplification was verified by agarose gelelectrophoresis.

Dot blot analysis

Dot blot analyses were carried out as previously described (Moter et al., 1998a). In brief, PCR products were spotted and cross-linked on a nylon membrane. Oligonucleotide probes (TRE I-VII) specific for the phylogenetic groups I - VII of oral treponemes were used as well as probes specific for DD-associated phylotypes DDKL-4, DDKL-12, DDKL-20 and for *T. brennaborense* and *T. socranskii*. Probes were labelled nonisotopically using the DIG labelling system (Boehringer Mannheim, Germany).

All hybridisation steps were performed at temperatures between 54 °C to 59 °C depending on the probe used. Stringency washes were performed with washing buffer containing 5x SSC and 0.1% SDS, 2x SSC and 0.1% SDS or 0,1x SSC and 0.1% SDS at temperatures ranging from 56 °C to 62 °C. Chemiluminescence detection was done with alkaline phosphatase labelled anti-DIG antibody and CSPD according to the manufacturer's instructions. After exposure to X-film (X-OMAT AR, Kodak, Germany) from 2 to 12 h the membrane was stripped with buffer containing 0,2 M NaOH and 0,1% SDS.

To assess specificity PCR amplification products of the 16S rRNA gene obtained from control strains listed below were included in all dot blot analyses.

Additionally, PCR products from a 16S rRNA gene clone library resembling uncultivated *Treponema* phylotypes groups I – VII of oral treponemes and DD-associated phylotypes DDKL-4, DDKL-12 and DDKL-20 were included as controls as described previously (Choi et al., 1997; Choi et al., 1994; Moter et al., 2006).

Fluorescence *in situ* hybridisation (FISH)

For FISH applications probes TRE I, TRE II, TRE IV and DDK-4 were used as previously described (Moter et al., 1998b). In brief, biopsies were fixed in 4% formaldehyde in PBS, pH 7.4 and embedded in Technovit 8100 (Heraeus Kultzer, Germany) according to the manufacturer's instructions. Histological sections (4 µm) were mounted on slides silanized with 3-aminopropyltrimethoxysilane (Sigma) and stored at 4°C.

FISH probes were synthesized commercially (Thermo Fisher Scientific, Germany) and 5`-labelled with Cy3 (indocarbocyanine) for the treponeme specific oligonucleotide probes TRE I, TRE II, TRE IV and DDK-4 while FITC (fluorescein isothiocyanate) was used for labelling the eubacterial probe EUB 338 as well as TRE I. FISH was performed at 46 °C in a humid chamber for 4 h using 20 µl hybridisation buffer for each section (20% deionised formamide, 0,9 M NaCl, 20 mM Tris-HCl pH 7.4, 0,01% SDS) and 100 ng of the probes. After incubation sections were washed with distilled water and mounted with ProLong® Antifade reagent (Molecular Probes, The Netherlands).

Fluorescent microscopy was performed using either an epifluorescence microscope (DMBL, Leica) or confocal laserscanning microscope (TCS SP2, Leica) for high resolution microscopy.

158 Control strains

159 The following strains were included as controls for dot blot hybridisation and FISH analyses:
160 *T. vincentii* ATCC 35580, *T. denticola* (ATCC 33521^T), T. *maltophilum* (ATCC 51939^T),
161 *T. lecithinolyticum* (ATCC 700332^T), *T. amylovorum* (ATCC 700288^T), *T. phagedenis*
162 (Biotype Reiter), *T. brennaborense* (DSM 12168^T), *T. socranskii* ssp. *socranskii* (ATCC
163 35536^T), *T. socranskii* ssp. *buccale* (ATCC 35534^T), *T. socranskii* ssp. *paredis* (ATCC
164 35535^T), *T. pectinovorum* (ATCC 33768^T).
165
166 Oligonucleotide sequences
167 Sequences of probes TRE I – VII, DDK-12, DDK-20 and TBREN and TSOC, specific for
168 *T. brennaborense* respectively *T. socranskii*, used in this study for dot blot and FISH analyses
169 have been described previously and have been deposited in ProbeBase, an online resource for
170 16S rRNA-targeted oligonucleotide probes where probe difference alignments are available
171 (Loy et al., 2003; Moter et al., 1998a; Schrank, 2000).
172 As previously described probe DDK-4 matches at position 1235 (in *E. coli* numbering) this
173 probe was not applicable for screening PCR products generated by SPU1 and RTU-3
174 amplifying only the first 550 bp of the 16S rRNA gene. For dot blot hybridisation analyses it
175 was therefore necessary to design a probe specific for *T. phagedenis*-like spirochetes
176 matching within the first 550 bases of the 16S rRNA gene. Specificity of this new designed
177 probe (5´-TCATCAAGGACGCATTCCCTCA-3´, position 460-481 in *E. coli* 16S rRNA
178 gene) for *T. phagedenis*-like spirochetes, designated DDK-4-B in the following, was checked
179 against all 16S rRNA gene sequences entries available in the Ribosomal Database Project II
180 (RDP) and the Genbank database using the software tool "Probe Match" which is part of the
181 RDP environment. Specificity of DDK-4-B in dot blot hybridisation analyses was ensured
182 additionally including representative 16S rRNA clones of above mentioned phylogenetic

183 groups of oral treponemes and DD associated phylotypes as well as several *Treponema*
184 control strains
185
186 **Results**
187 Dot blot hybridization
188 Using the newly designed probe DDK-4-B specific for *T. phagedenis*-like spirochetes in dot
189 blot hybridization analyses positive results were obtained only for *T. phagedenis* and
190 phylotype DDKL-4 while the other control strains remained negative proving specificity of
191 this probe.
192 Using primer SPU1/RTU3 amplification of the partial 16S rRNA gene was successful in all
193 samples resulting in PCR products of about 550 bp. Dot blot analyses with probes specific for
194 the phylogenetic groups I, II and IV of oral treponemes gave positive results in up to 84 % of
195 the samples while probes specific for phylogenetic groups III, V, VI and VII of oral
196 treponemes and specific for *T. socranskii* remained negative in all samples. When
197 oligonucleotide probes specific for *T. phagedenis*-like spirochetes and DD-associated
198 phylotypes DDKL-12 and DDKL-20 as well as specific for *T. brennaborense* were applied,
199 positive results were obtained only for *T. phagedenis*-like spirochetes and phylotype DDKL-
200 12 while phylotype DDKL-20 and *T. brennaborense* could not be detected by dot blot
201 hybridization.
202 *T. phagedenis*-like spirochetes were most prevalent und could be detected in 100 % of the
203 samples. DDKL-12, in contrast, was detectable only in 66.1 %. Somewhat more prevalent
204 was phylogenetic group I with 83.9 %, phylogenetic group II with 80.4 % and phylogenetic
205 group IV of oral treponemes with 82.1 % (Fig.1).
206 Most of the samples were found positive for several phylogenetic groups and phylotypes
207 simultaneously, summed up as treponemal groups in the following (Tab. 1). In 46.4 % of the
208 samples five different treponemal groups were discovered simultaneously, while in 34.0 % of

the samples four different treponemal groups are still detectable. To a much lesser extent only three respectively two treponemal groups are present in 7.2 % respectively 12.4 % of the samples.

These treponemal groups detected in DD lesions could be assigned to ten different combinations of treponemal groups (Tab.1). Among these combinations phylotype groups I, II, IV and phylotypes DDKL-4 and DDKL-12 or the combination of phylotype groups I, II, IV and phylotype DDKL-4 were most frequently detected in 46.4 % respectively 25.0 % of the samples. Based on these data, it is obviously that at least phylotype groups I, II, IV and phylotypes DDKL-4 are most common in DD being detectable in nearly 71 % of the samples.

For visualizing the spatial distribution of treponemes in DD affected tissue five biopsies already tested positive by dot blot hybridization for phylotype groups I, II and IV and phylotype DDKL-4 were examined by FISH analyses (Fig. 2). As shown by these specific oligonucleotide probes in combination with the eubacterial probe EUB338 *Treponema* spp. represent a high proportion of the bacterial mass.

Furthermore, treponemes were not only detectable in superficial layers of the epidermis but also in deeper tissue layers. Mainly phylotype DDKL-4 and phylotype group I tend to spread in great numbers even into deeper and healthy layers of the epidermis while treponemes of phylotype group IV and II occurred to a lesser extent within the epidermis. FISH also revealed that treponemes invade the tissue penetrating yet uninfected tissue via intracellular junctions (Fig. 2)

Discussion

Recent investigations based on 16S rRNA sequence analyses revealed a high diversity of treponemes associated with DD (Choi et al., 1994; Trott et al., 2003). As cultivation of treponemes is highly sophisticated routine bacteriological techniques are presently insufficient for identification of treponemes in diagnostic samples.

Therefore dot blot hybridisation assays with a panel of treponeme specific probes were used to assess most comprehensive the treponemal epidemiology by screening a large number (n=56) of DD samples. For detection of *T. phagedenis*-like spirochetes a new probe, designated DDK-4-B, was designed and evaluated in this study for use in dot blot hybridization analysis showing specificity only for *T. phagedenis*-like treponemes.

By this approach we could identify several treponemal groups in DD samples. Most frequently treponemes belonging to the *T. phagedenis*-like group (100 %) were identified followed by the phylogenetic groups TRE I (83.9 %), TRE IV (82.1 %), TRE II (80.4 %) while phylotype DDKL-12 was detected only in 66.1 % (Fig.1) being consistent with recent studies reporting successful cultivation and characterization of *T. vincentii*-like and *T. denticola*-like treponemes which are cultivable representative species of the phylogenetic groups TRE I and TRE II as well as the frequent isolation of *T. phagedenis*-like treponemes from DD-lesions (Demirkan et al., 1999; Stamm et al., 2002; Walker et al., 1995).

Additionally, in this study treponemes of group TRE IV and the DD associated phylotype DDKL-12 were detected in a large number of samples (82.1 % respectively 66.1 %) by dot blot hybridization. So far, no strain of both groups was successfully isolated from DD lesions by cultivation. Only previously studies reported the detection of these treponemes by cultivation-independent methods (Choi et al., 1994; Moter et al., 1998b).Theses findings are most probably due to the fastidious character of treponemes during cultivation and the difficulty in obtaining single *Treponema* isolates from samples associated with a mixed polymicrobial flora like those associated with DD samples.

As the combination of TRE I - TRE II - TRE IV –DDKL-4 was present in up to 71 % of all samples investigated, this microbial community seems to be the most important one for the development of DD. Similar to recent findings that different bovine strains of *T. phagedenis*-like spirochetes are not only antigenetic but also pathogenetically different it is also conceivable that certain combinations of *Treponema* sp. might be more pathogenic than

261 others and are therefore favoured in the course of infection maybe due to synergistic effects of
262 yet rarely known virulence or fitness mechanisms of these treponemes (Elliott et al., 2007).
263 This underlines the complexity of DD and the need to rule out further virulence traits of
264 bovine *Treponema* strains as it already has done in recent studies (Edwards et al., 2003b;
265 Elliott et al., 2007; Zuerner et al., 2007).
266 In contrast to those treponemes mentioned above, the DD associated phylotype DDKL-20 and
267 *T. brennaborense*, previously isolated from a DD sample, could not be detected in any of
268 these DD samples. One reason for these negative dot blot results might be that phylotype
269 DDKL-20 as well as *T. brennaborense* occur only in very low numbers in DD lesions below
270 the detection limit of the techniques used. It is also possible that these treponemes only occur
271 very sporadically in DD lesions and are rather environmental species like *T. bryantii* which is
272 an apathogenic treponeme belonging to the normal bacterial flora of the bovine rumen
273 (Stanton and Canale-Parola, 1980). Consequently, the significance of these treponemes in the
274 pathogenesis of DD remains questionable.
275 Similarly, phylogenetic groups III, V, VI and VII as well as *T. socranskii* could not be
276 detected in our study indicating that these treponemes that were originally derived from the
277 human oral cavity are simply not present in DD lesions or they are also not detectable by dot
278 blot analyses due to very low numbers remaining their function in the pathogenesis of DD
279 questionable.
280 Regarding dot blot and FISH results the most important *Treponema* spp. among the different
281 *Treponema* groups detected in DD lesions seem to be the *T. phagedenis*-like group and
282 treponemes belonging to the phylogenetic group I of oral treponemes. By FISH analyses these
283 treponemes can not only be found in a high number of samples but also in deeper tissue layers
284 at the transition of healthy and diseased tissue. FISH revealed that these treponemes are also
285 present in very high numbers compared to the total bacterial count as demonstrated by the use
286 of the universal bacterial probe EUB338. Our results underline that these treponemes have a

major pathogenic function in the aetiology of DD, also supported by recent findings suggesting that bovine *T. phagedenis*-like spirochetes impair the innate immune response and wound repair functions and resist clearance from infected tissue (Elliott et al., 2007).

Further treponemes frequently detected by dot blot hybridisation are those belonging to phylogenetic group TRE II and TRE IV. FISH analyses revealed that these treponemes occur mostly only in lower numbers similarly to results from Moter et al. (1998b). Therefore these treponemes are most likely not as important as treponemes of group TRE I and the *T. phagedenis* –like group.

Although phylotype DDKL-12 can be detected in 63.8 % of the samples its function in the aetiology of DD remains undetermined. As no cultivable *Treponema* sp. is available to serve as a validate control strain for evaluating FISH conditions such analyses have not been performed so far. Therefore no data about the spatial distribution and the quantity in DD lesion exist remaining the rule of this phylotype unclear.

Screening a large number of samples our results clearly indicate that DD is usually characterized by a high prevalence and diversity of *Treponema* spp. associated with DD. Furthermore, our dot blot hybridization and FISH analyses clearly suppose *Treponema* spp. to be among the most important agents in the pathogenesis of DD. Therefore future work has to be focused on the epidemiology of *Treponema* spp. associated with DD, how infection by *Treponema* spp. is initiated and on virulence traits being important for causing and maintaining DD.

References

Cheli, R., Mortellaro, C.M., 1974. La dermatite digitale del bovino. In: Int. Conf. Diseases of Cattle, Milan, Italy, pp. 208-213.

Choi, B.K., Nattermann, H., Grund, S., Haider, W., Gobel, U.B., 1997, Spirochetes from digital dermatitis lesions in cattle are closely related to treponemes associated with human periodontitis. Int J Syst Bacteriol 47, 175-181.

Choi, B.K., Paster, B.J., Dewhirst, F.E., Gobel, U.B., 1994, Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect Immun 62, 1889-1895.

Cruz, C.E., Pescador, C.A., Nakajima, Y., Driemeier, D., 2005, Immunopathological investigations on bovine digital epidermitis. Vet Rec 157, 834-840.

Demirkan, I., Carter, S.D., Hart, C.A., Woodward, M.J., 1999, Isolation and cultivation of a spirochaete from bovine digital dermatitis. Vet Rec 145, 497-498.

Dhawi, A., Hart, C.A., Demirkan, I., Davies, I.H., Carter, S.D., 2005, Bovine digital dermatitis and severe virulent ovine foot rot: a common spirochaetal pathogenesis. Vet J 169, 232-241.

Edwards, A.M., Dymock, D., Jenkinson, H.F., 2003a, From tooth to hoof: treponemes in tissue-destructive diseases. J Appl Microbiol 94, 767-780.

Edwards, A.M., Dymock, D., Woodward, M.J., Jenkinson, H.F., 2003b, Genetic relatedness and phenotypic characteristics of Treponema associated with human periodontal tissues and ruminant foot disease. Microbiology 149, 1083-1093.

Elliott, M.K., Alt, D.P., Zuerner, R.L., 2007, Lesion formation and antibody response induced by papillomatous digital dermatitis-associated spirochetes in a murine abscess model. Infect Immun 75, 4400-4408.

Grund, S., Nattermann, H., Horsch, F., 1995, [Electron microscopic detection of spirochetes in dermatitis digitalis of cattle]. Zentralbl Veterinarmed [B] 42, 533-542.

Logue, D.N., Offer, J.E., Laven, R.A., Ellis, W.A., 2005, Digital dermatitis--the aetiological soup. Vet J 170, 12-13.

Loy, A., Horn, M., Wagner, M., 2003, probeBase: an online resource for rRNA-targeted oligonucleotide probes. Nucleic Acids Res 31, 514-516.

Moter, A., Hoenig, C., Choi, B.K., Riep, B., Gobel, U.B., 1998a, Molecular epidemiology of oral treponemes associated with periodontal disease. J Clin Microbiol 36, 1399-1403.

Moter, A., Leist, G., Rudolph, R., Schrank, K., Choi, B.K., Wagner, M., Gobel, U.B., 1998b, Fluorescence in situ hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions. Microbiology 144, 2459-2467.

Moter, A., Riep, B., Haban, V., Heuner, K., Siebert, G., Berning, M., Wyss, C., Ehmke, B., Flemmig, T.F., Gobel, U.B., 2006, Molecular epidemiology of oral treponemes in patients with periodontitis and in periodontitis-resistant subjects. J Clin Microbiol 44, 3078-3085.

Nattermann, H., Grund, S., Ehrlich, M., Zucker, B., Choi, B.-K., Göbel, U.B., 1998. Zur Ätiologie und Immunprophylaxe der Dermatitis digitalis des Rindes. In: Proc. 10th Middle-European Buiatrics Congress, Siófok, pp. 74-84.

Nordhoff, M., Wieler, L.H., 2005, [Incidence and significance of treponemes in animals]. Berl Munch Tierarztl Wochenschr 118, 24-36.

Rodriguez-Lainz, A., Melendez-Retamal, P., Hird, D.W., Read, D.H., Walker, R.L., 1999, Farm- and host-level risk factors for papillomatous digital dermatitis in Chilean dairy cattle. Prev Vet Med 42, 87-97.

Schlafer, S., Nordhoff, M., Wyss, C., Strub, S., Hubner, J., Gescher, D.M., Petrich, A., Gobel, U.B., Moter, A., 2008, Involvement of Guggenheimella bovis in digital dermatitis lesions of dairy cows. Vet Microbiol 128, 118-125.

Schrank, K., 2000. Diversity of treponemes from Dermatitis digitalis of cattle- description of novel species Treponema brennaborense. PhD thesis. Freie Universität, Berlin.

Schrank, K., Choi, B.K., Grund, S., Moter, A., Heuner, K., Nattermann, H., Gobel, U.B., 1999, Treponema brennaborense sp. nov., a novel spirochaete isolated from a dairy cow suffering from digital dermatitis. Int J Syst Bacteriol 49 Pt 1, 43-50.

Stamm, L.V., Bergen, H.L., Walker, R.L., 2002, Molecular Typing of Papillomatous Digital Dermatitis-Associated Treponema Isolates Based on Analysis of 16S-23S Ribosomal DNA Intergenic Spacer Regions. J Clin Microbiol 40, 3463-3469.

Stanton, T.B., Canale-Parola, E., 1980, Treponema bryantii sp. nov., a rumen spirochete that interacts with cellulolytic bacteria. Arch Microbiol 127, 145-156.

Trott, D.J., Moeller, M.R., Zuerner, R.L., Goff, J.P., Waters, W.R., Alt, D.P., Walker, R.L., Wannemuehler, M.J., 2003, Characterization of Treponema phagedenis-like spirochetes isolated from papillomatous digital dermatitis lesions in dairy cattle. J Clin Microbiol 41, 2522-2529.

Walker, R.L., Read, D.H., Loretz, K.J., Nordhausen, R.W., 1995, Spirochetes isolated from dairy cattle with papillomatous digital dermatitis and interdigital dermatitis. Vet Microbiol 47, 343-355.

Wells, S.J., Garber, L.P., Wagner, B.A., 1999, Papillomatous digital dermatitis and associated risk factors in US dairy herds. Prev Vet Med 38, 11-24.

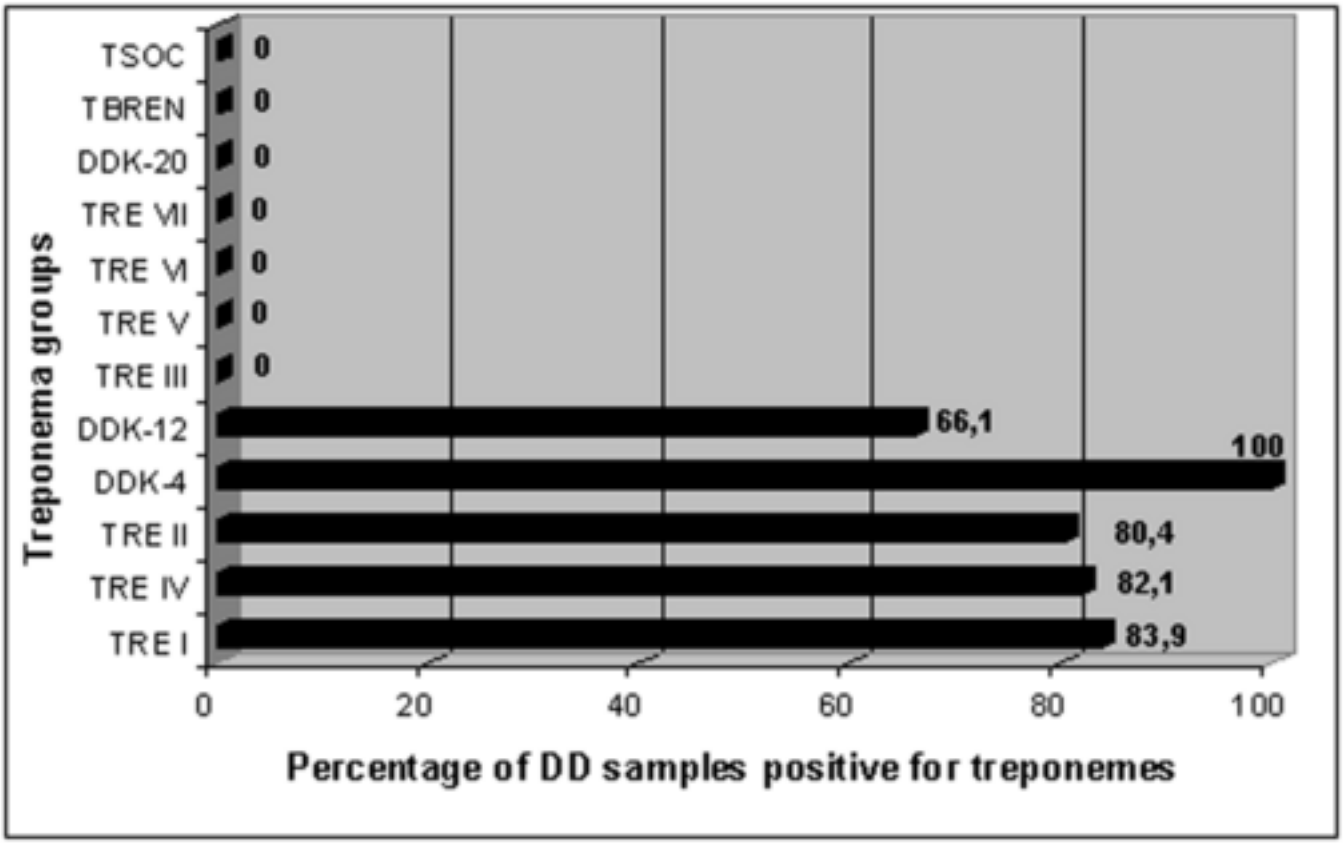
Wyss, C., Dewhirst, F.E., Paster, B.J., Thurnheer, T., Luginbuhl, A., 2005, Guggenheimella bovis gen. nov., sp. nov., isolated from lesions of bovine dermatitis digitalis. Int J Syst Evol Microbiol 55, 667-671.

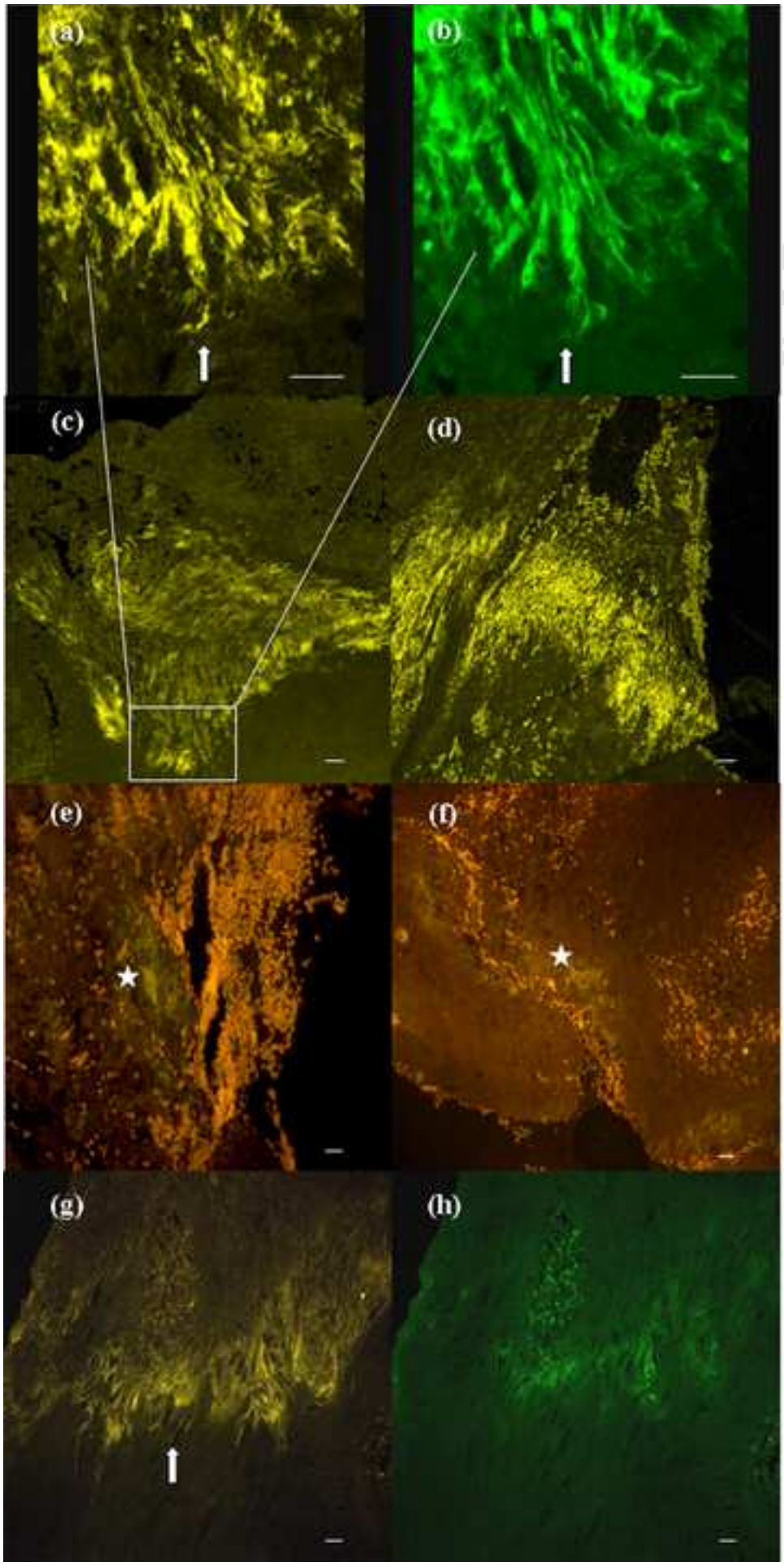
Zuerner, R.L., Heidari, M., Elliott, M.K., Alt, D.P., Neill, J.D., 2007, Papillomatous digital dermatitis spirochetes suppress the bovine macrophage innate immune response. Vet Microbiol.

Fig. 1: Percentage of positive samples (total number = 56) detected by dot blot hybridization using *Treponema* specific oligonucleotide probes.

Tab.1: Detailed distribution patterns of *Treponema* groups in biopsies. In most of the samples at least four different *Treponema* groups can be detected simultaneously indicating a very high diversity of *Treponema* spp. in digital dermatitis.

Fig. 2: FISH analyses of DD-bioptates using *Treponema*-specific oligonucleotides.
Fluorescence *in situ* hybridisation of DD sections at high magnification with (a) DDK-4 (yellow) and the (b) universal bacterial probe EUB338 (green) respectively at (c) magnification. Hybridisation with (a, c) DDK-4 (yellow) and (d) TRE-I (yellow) show large amounts of treponemes invading deep layers of the epidermis.
In contrast treponemes (yellow) belonging to phylogenetic group II (e) and phylogenetic group IV (f) are only present in lower numbers as indicated by asterisks.
Simultaneous hybridisation with (g) DDKL-4 (yellow) and (h) TRE-I (green).
Arrows indicate treponemes located at the interface of healthy and affected tissue.
Bar: 20 µm.





number of different treponemal groups	detailed composition of. *Treponema* spp.	prevalence (n = 56)
5 treponemal groups:	TRE I - TREII - TREIV- DKL-4 - DDKL -12	46.4 %
	total:	**46.4 %**
4 treponemal groups:	TRE I - TRE II - TRE IV - DDKL-4	25.0 %
	TRE I - TRE IV - DDKL-4 - DDKL-12	3.6 %
	TRE I - TRE II - DDKL-4 - DDKL-12	3.6 %
	TRE II - TRE IV - DDKL-4 - DDKL-12	1.8 %
	total:	**34,0 %**
3 treponemal groups:	TRE I - TRE IV - DDKL-4	3.6 %
	TRE IV - DDKL-4 - DDKL-12	1.8 %
	TRE I - DDKL-4 - DDKL-12	1.8 %
	total:	**7.2 %**
2 treponemal groups:	DDKL-4 - DDKL-12	8.8 %
	TRE II - DDKL-4	3.6 %
	total:	**12.4 %**