

## Culture and molecular identification of microorganisms from Digital Dermatitis lesions in dairy cattle: *Leptospira*, an unexpected finding

[Cultivo e identificação molecular de microrganismos isolados de lesões de dermatite digital em bovinos leiteiros: *Leptospira*, um achado inesperado]

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### ABSTRACT

Bovine digital dermatitis (BDD) is an infectious and contagious disease characterized by ulcerative and proliferative lesions affecting the skin on the bulbs of the heel or the interdigital cleft in dairy cattle, often associated with lameness. Evidences on the etiology of BDD indicate that it is multifactorial, involving environmental factors and multiple bacterial colonization. We isolated and identified microorganisms from BDD biopsy samples obtained from five Holstein Friesian and two Jersey cows by cultivation and molecular identification of bacterial isolates using 16S rRNA gene sequence analysis. We identified six bacterial species: Spirochetes as *Treponema pedis* and *Leptospira broomi/L. fainei*, *L. licerasiae/L. wolffii*; *Corynebacterium appendicis*, *Cupriavidus gilardii* and *Enterococcus casseliflavus/E. gallinarum*. It was quite surprising to have isolated and identified *Leptospira* species in three out of seven cultures, from different individual cows and two different farms. The species identified belong to the intermediate pathogenic clade, which is a group found to cause human and animal disease. Our findings indicate the need to further investigate the association of *Leptospira* of intermediate pathogenicity with BDD lesions and whether its presence would have any veterinary and medical significance both in Leptospirosis and with the pathogenesis of BDD lesions, especially in tropical countries

**Keywords:** bovine digital dermatitis; molecular identification; 16S rRNA gene analysis; *Treponema*; *Leptospira*

### RESUMO

*Dermatite digital bovina (DDB) é uma doença infecciosa, contagiosa, caracterizada por lesões ulcerativas e proliferativas da região dos talões e/ou do espaço interdigital, frequentemente associada com claudicação. Evidências indicam que a etiologia da DDB é multifatorial, envolvendo fatores ambientais e colonização polimicrobiana. Relata-se aqui o isolamento e a identificação bacteriana em amostras de biópsias em lesões de DDB, obtidas de cinco vacas da raça Holandesa e duas da raça Jersey, por meio de cultivo e identificação molecular de isolados, com base na análise de sequências de genes 16S rRNA. São identificadas seis espécies bacterianas: as espiroquetas *Treponema pedis* e *Leptospira broomi/L. fainei*, *L. licerasiae/L. wolffii*; *Corynebacterium appendicis*, *Cupriavidus gilardii* e *Enterococcus casseliflavus/E. gallinarum*. O isolamento e a identificação de espécies de *Leptospira* surpreenderam, destacando-se sua presença em três dos sete cultivos obtidos em diferentes vacas, de duas fazendas distintas. As espécies identificadas pertencem ao grupo tipificado como de patogenicidade intermediária, causador de doenças em animais e no homem. Os resultados apresentados indicam a necessidade de maiores investigações sobre a associação entre *Leptospira* de patogenicidade*

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*intermediária e a patogênese das lesões DDB, investigando-se sua presença e significado nas medicinas veterinária e humana, especialmente em países tropicais.*

*Palavras-chave: dermatite digital bovina; identificação molecular; análise do gene 16S rRNA; Treponema; Leptospira*

## INTRODUCTION

Bovine Digital Dermatitis (BDD), or Mortellaro's disease (Krull *et al.*, 2015), is an infectious and contagious disease characterized by ulcerative and proliferative lesions of the foot skin in dairy cattle and is often associated with lameness. The disease can result in economic losses having a negative impact on reproductive efficiency, productive life span, welfare, and milk production (Walker *et al.*, 1995, Fidler *et al.*, 2012). BDD has been reported in several European countries, Canada, United States, Mexico, Japan, and in some South American countries like Chile and Brazil (Molina *et al.*, 1999, Nascimento *et al.*, 2015).

The etiology of BDD is not well established, but it is believed to be multifactorial, involving environmental management and physiological and microbiological causes (Klitgaard *et al.*, 2008; Zinicola *et al.*, 2015). Previous studies have demonstrated that multiple bacterial species are associated with lesions, with spirochetes being the most reliably identified organisms (Krull *et al.*, 2015). However, the task of clarifying the etiology of BDD is very difficult because those spirochetes are notoriously difficult to cultivate. Consequently, culture-independent phylogenetic methods, such as comparative 16S rRNA gene sequence analysis of BDD lesions, are a valuable instrument in determining the diversity, phylogeny, prevalence and spatial distribution of unclassified BDD spirochetes (Klitgaard *et al.*, 2008). Nevertheless, this method is unable to provide specific information about the physiology of these microorganisms, meaning that it is necessary to make use of culture-dependent approaches.

In this work, we evaluate the presence of bacterial populations, with emphasis on the spirochetes by cultivation, isolation and molecular identification of strains from dairy-cow lesions spontaneously affected by BDD.

## MATERIALS AND METHODS

Biopsy samples were obtained from five Holstein Friesian and two Jersey cows with BDD lesions. The sample collection was performed by a veterinarian in two dairy farms around of the city of Belo Horizonte, Minas Gerais state, Brazil. The feet of cows were washed with water and scrubbed with a soft brush to remove soil and other particles attached to the skin surface. Lidocaine 2% (5mL) was injected subcutaneously around the lesion site and skin biopsy specimens, fragments of 6mm, were taken from ulcerative areas of each lesion, using a sterile biopsy punch. The biopsy samples were placed in 2mL of sterile pre-reduced peptone yeast glucose broth (PRAS-PYG) and anaerobically transported to the laboratory. After the biopsies were collected, the seven cows were treated by the veterinarian. Research was approved by the Ethics Committee in Animal Experimentation of Universidade Federal de Minas Gerais (CEUA/UFMG), registration number 121 in 2015.

To verify the presence of microorganisms, the direct examination of biopsy was conducted by observing Gram-stained smears from the broth and, for spiraled microorganisms, observations were made through dark field microscopy (Walker *et al.*, 1995).

Next, samples were mechanically dispersed and homogenized in PRAS-PYG broth, with 200µL of this suspension being used to inoculate 5.0mL of anaerobic oral *Treponema* isolation broth (OTI) with rifampin 1µg/mL and enrofloxacin 5µg/mL and to inoculate 5.0mL of *Leptospira* Medium Base Ellinghausen and McCullough modified by Johnson and Harris (EMJH) (Difco, BD Biosciences), supplemented with bovine albumin (Johnson and Faine, 1984) plus 5µg/mL enrofloxacin. To cultivate *Leptospira*, incubation was at 28°C and 37°C for up to 4 weeks in microaerophilic and anaerobic conditions. When initial growth was perceived, other replicas were made in EMJH medium.

DNA was isolated from cultivated bacteria. The entire cultures of each sample (3mL) were centrifuged (8000 x g, 10min) and genomic DNA was obtained using the Wizard Genomic DNA Purification Kit (Promega) according to recommendations of the manufacturer.

Species-level identification was performed by 16S rRNA gene sequencing and sequencing analysis. PCR reactions were performed in 50µL total volume containing 30 pmols of each 27F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' GGT TAC CTT GTT ACG ACT T 3') primers, described by Lane (1991), 10ng of DNA template and PCR Master Mix (Promega Corporation, Madison, USA) with 200µM dNTPs, 1.5mM MgCl<sub>2</sub> and 1.5 units *Taq* DNA polymerase. Amplification occurred over 35 cycles (95°C for 30 s, 55°C for 1 min and 72°C for 1 min) after an initial denaturation (95°C for 2 min) and completed by a final extension (72°C for 5 min). Negative and positive controls were included. The amplicons were purified using the Wizard® SV Gel and PCR Clean-up System kit (Promega Corporation, Madison, USA) and sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Piscataway, USA) for the system automatic sequencing of MegaBACE 1000 (GE Healthcare, Piscataway, USA).

Forward and reverse sequence reads were assembled using the Phred-Phrap-Consed package with default parameters. The consensus sequence obtained was compared with those deposited in the GenBank database using the BLASTn algorithm v.2.2.15, available on the website of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast>), to find a match to nearly identical sequences (>97% identity) for the 16S rRNA.

The phylogenetic analyses were performed using MEGA 6.06 software with default parameters (Tamura K. *et. al* 2013). Dataset sequences of 16S rRNA of type strains belonging to *Corynebacterium*, *Cupriavidus*, *Enterococcus*, *Leptospira* and *Treponema* genera were obtained from GenBank (supplementary material) and used to perform a multiple sequence alignment

using ClustalW software. Next, the best mutational model was calculated for each genus, which was used as a parameter for phylogenetic inferences (evolutionary model of nucleic acids) by the Maximum likelihood method (Hall, 2013).

## RESULTS

Preparations of all biopsies were examined in dark-field microscopy for the presence of spirochetes, and smears were also observed through light microscopy after Gram staining before and after cultivating. Spirochetes were observed in four biopsy suspensions (A8T, 6MC, 7MC, and 8MC). In addition, short rods (4MC, 9MC) and cocci (5MC) were detected (Tab. 1)

All seven biopsy samples became positive in the cultures. The 16S rRNA gene PCR amplified products from each of the seven bacterial isolates were sequenced. The partial nucleotide sequences obtained (average of 760 base pairs) were analyzed by BLASTN searches in order to compare our consensus sequences against specific 16S ribosomal RNA (Bacteria and Archaea) database at the NCBI website. The number of nucleotides sequenced, the highest similarities found for each sample and the most probable species are indicated in (Tab. 1).

Pure spirochete cultures were obtained from four cows. Comparison of the bacterial 16S rRNA gene sequences from samples A8T, 7MC and 8MC by BLASTN searches showed 99% identity with members of the *Leptospira* genus. The sequences from samples A8T and 8MC had best hits with *L. broomi* and *L. fainei* serovar Hurstbridge. The sequence from sample 7MC had best hits with *L. licerasiae* serovar Varillal and *L. wolffii* serovar Khorat (Tab. 1).

The Figure 1 shows the molecular phylogenetic analysis by maximum likelihood of the *Leptospira* sequences obtained from pure spirochete cultures. The fourth spirochete positive sample was 6MC and the sequence obtained showed the best hit with *Treponema pedis* (98%), as confirmed by the molecular phylogenetic analysis shown in Figure 2.

Table 1. Molecular identification of bacterial isolates using 16S rRNA gene sequencing and sequences analysis

Biopsy sample	Microscopy	Nucleotide Length (pb)	Genebank accession number	Identity (%)	Species
A8T	Spirochetes	766	gi 55832798 gi 54112246	99.0	<i>Leptospira broomii</i> ATCC BAA-1107 or <i>L.fainei</i> serovar Hurstbridege ATCC BAA-1109
4MC	Rod	780	gi 22265710	99.5	<i>Corynebacterium appendicis</i> DSM 44531T
5MC	Cocci	888	gi 2828136  gi 2828133	99.0	<i>Enterococcus casseliflavus</i> ATCC 25788 or <i>Enterococcus gallinarum</i> ATCC 49573
6MC	Spirochetes	498	gi 116805562	98.0	<i>Treponema pedis</i> DSM 18691
7MC	Spirochetes	794	gi 148733589 gi 117415055	99.0	<i>Leptospira licerasiae</i> ATCC BAA-1110 serovar varillal or <i>L. wolffii</i> serovar Khorat Khorat-H2
8MC	Spirochetes	825	gi 55832798 gi 54112246	99.0	<i>Leptospira broomii</i> ATCC BAA-1107 or <i>L. fainei</i> serovar Hurstbridege ATCC BAA-1109
9MC	Rod	803	gi 3643825	99.0	<i>Cupriavidus gilardii</i> ATCC 700815

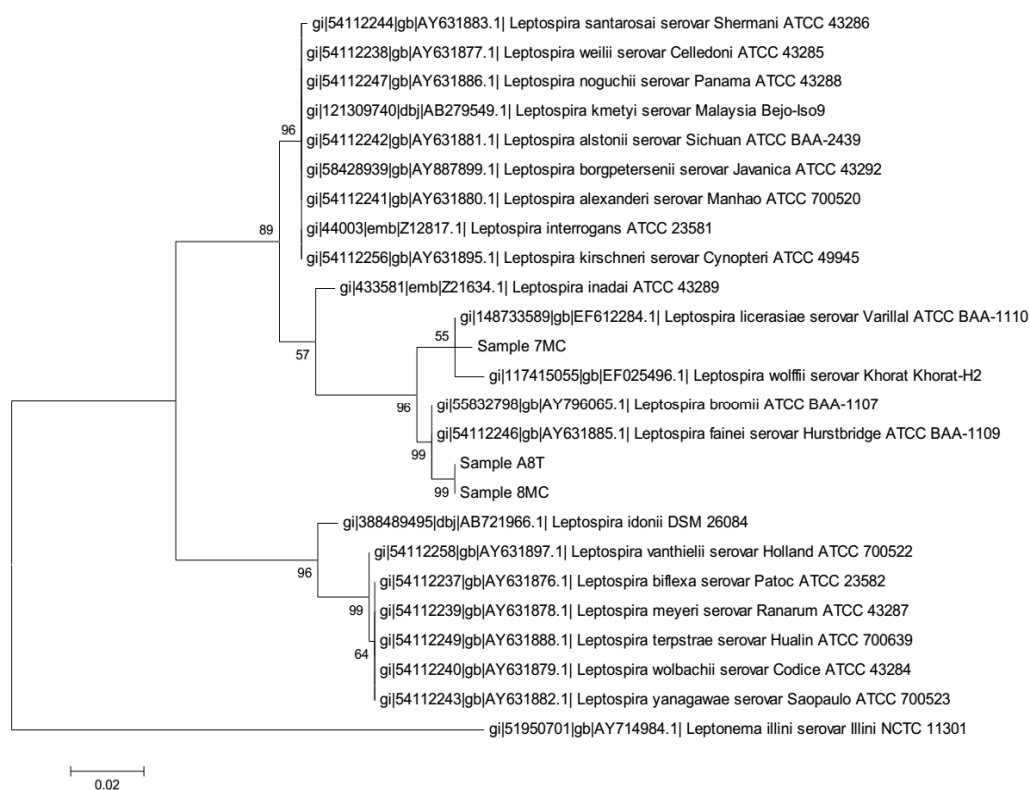


Figure 1. Molecular Phylogenetic analysis by maximum likelihood method of three pure spirochete cultures (samples 7MC, A8T, 8MC) for identification as *Lepstospira*. The sequences from samples A8T and 8MC had best hits with *L. broomi* and *L. fainei* serovar Hurstbridge. The sequence from sample 7MC had best hits with *L. licerasiae* serovar Varillal and *L. wolffii* serovar Khorat.

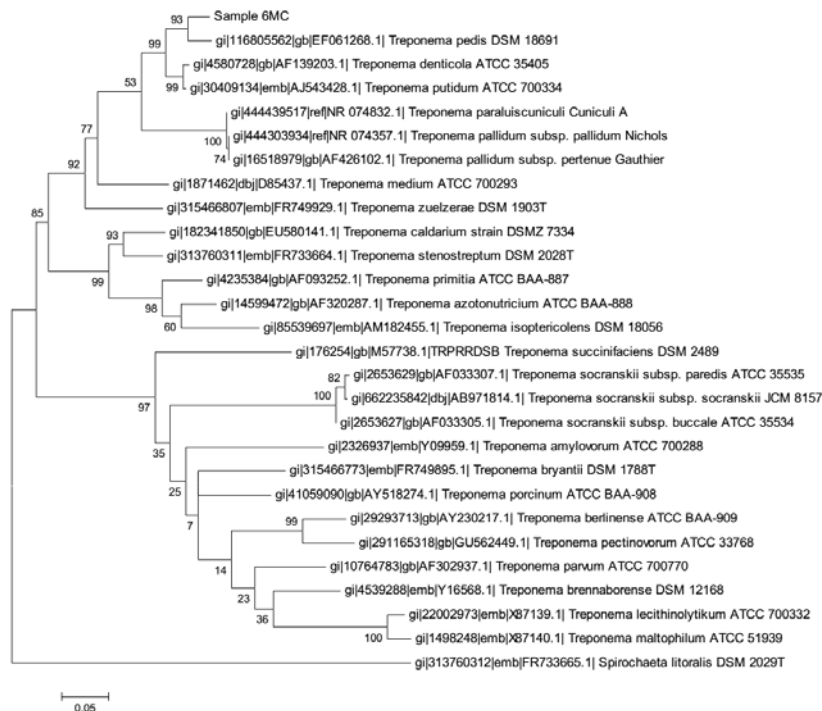


Figure 2. Molecular Phylogenetic analysis by maximum likelihood method for molecular identification of a pure culture sample 6MC as *Treponema pedis*.

From sample 4MC, the bacterial 16S rRNA gene sequences from pure culture showed the highest similarity with *Corynebacterium appendicis* (99.5%) and the corresponding phylogenetic tree is presented in Figure 3. From sample 5MC, the sequences with the best hits indicated the genus *Enterococcus*, and the species *Enterococcus casseliflavus* and *E. gallinarum* presenting the highest similarity (99%), as shown in the phylogenetic tree in Figure 4. Also, the sequences obtained from sample 9MC showed the best hits with *Cupriavidus gilardii* (99%) and the corresponding phylogenetic tree is presented in Figure 5.

## DISCUSSION

According to Krull *et al.* (2015), sequencing of DNA from 48 biopsies confirmed the presence of complex communities composed of one hundred bacterial species present in the lesions. Treponemes and *Corynebacteriaceae* were among the top 12 bacterial species for each lesion stage. In the present work, both Treponemes and *Corynebacteriaceae* were isolated and identified from cultures derived

from samples 6MC and 4MC (Table 1), respectively, as *T. pedis* and *C. appendicis*. Evans *et al.*, 2009, describes *Treponema pedis* sp.nov. a spirochaete representing a novel species of the genus *Treponema* isolated from bovine digital dermatitis lesions. The novel isolates form a coherent *Treponema* sp. taxonomic group. In the USA, 99.0% of the samples isolated from BDD lesions were identified as *T. pedis* sp. nov. strains by 16S rRNA gene sequence similarity, (Walker *et al.*,1995). This possibly indicates worldwide (UK, USA, Germany ) presence of *T. pedis* sp.nov. suggesting that this microorganism might have a significant etiological role in BDD. On the other hand, BDD appears to have a complicated etiology involving co-infection by several different treponemes (Choin *et al.*, 1997). The presence of a diverse range of treponemes in polytreponemally infected tissue has been identified by cloning and sequencing of 16S rRNA genes, but their highly fastidious nature has, for the most part, precluded any further characterization of these organisms (Evans *et al.*, 2009).

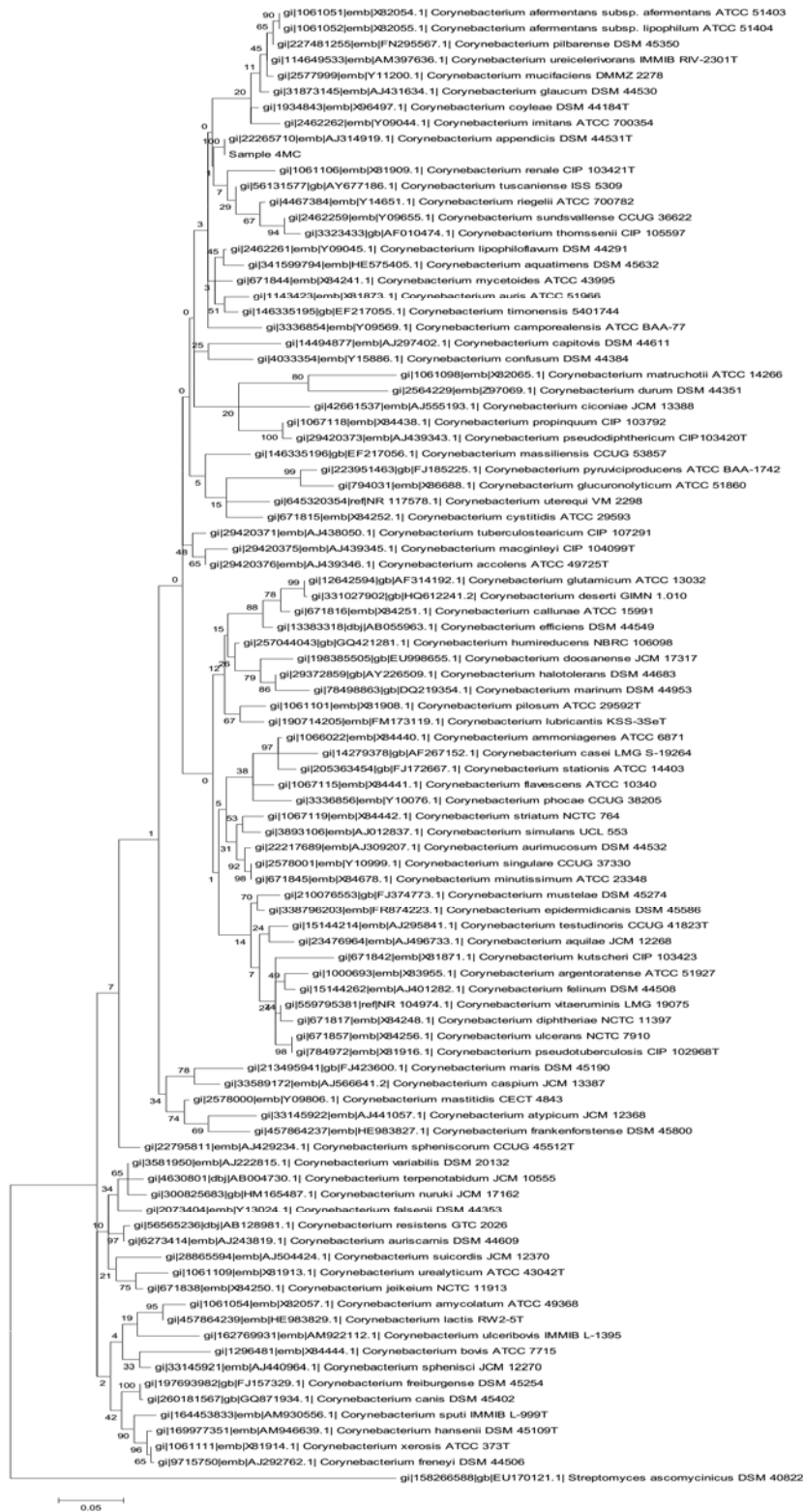


Figure 3. Molecular Phylogenetic analysis by Maximum Likelihood method for molecular identification of a pure culture (sample 4MC) as *Corynebacterium appendicis*.

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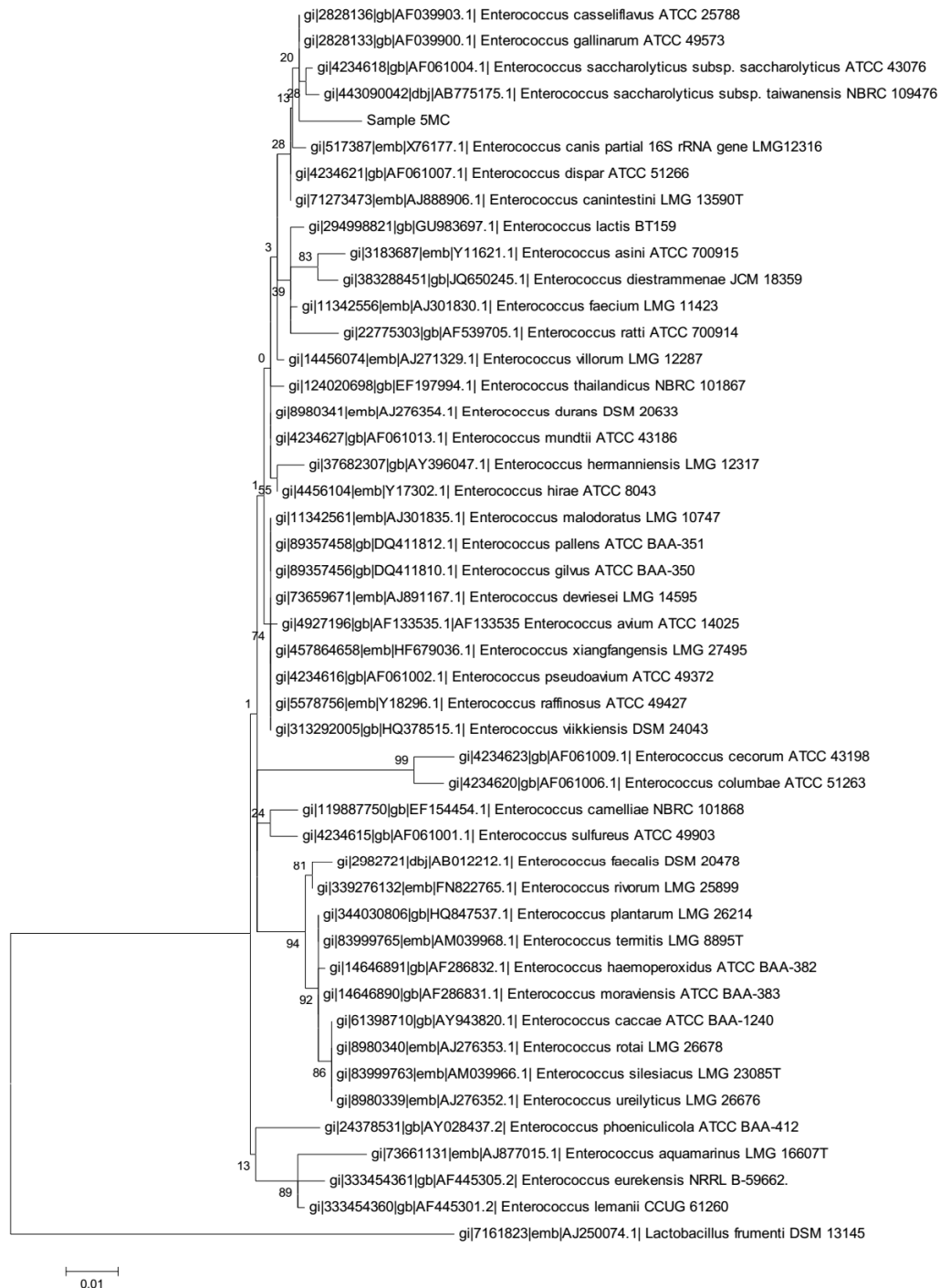


Figure 4. Molecular Phylogenetic analysis by Maximum Likelihood method for identification of the pure culture corresponding to sample 5MC as *Enterococcus casseliflavus* and *E. gallinarum*.

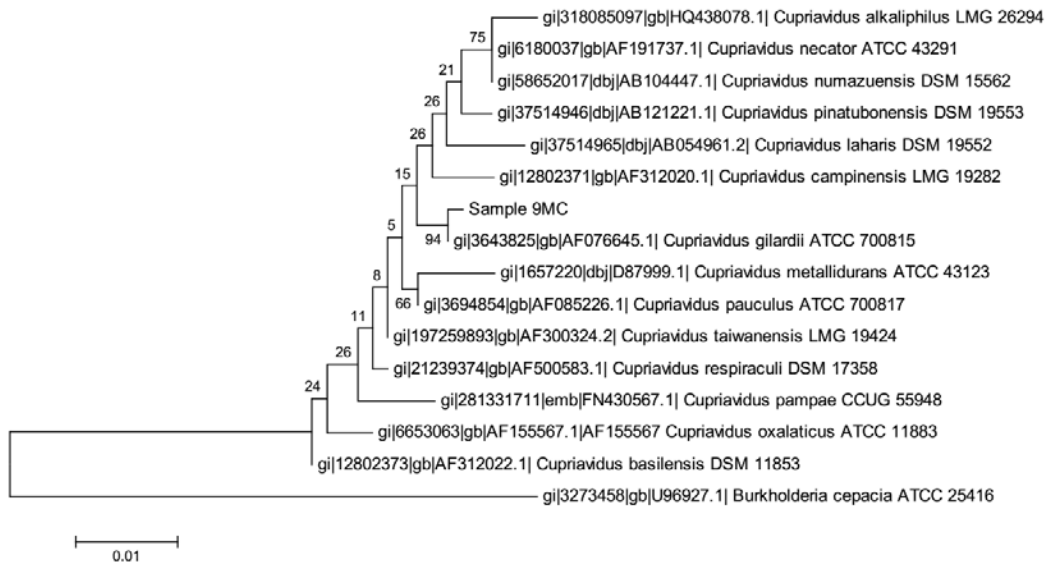


Figure 5. Molecular Phylogenetic analysis by Maximum Likelihood method for identification of the pure culture corresponding to sample 9MC as *Cupriavidus gilardii*.

*C. appendicis*, a species proposed by Yassin *et al.* (2002), is a facultative anaerobe that was previously isolated from abdominal swabs of a human patient with appendicitis accompanied with abscess formation. The culture derived from sample 5MC had the best hits with *Enterococcus casseliflavus* and *Enterococcus gallinarum*. The *Enterococci* are Firmicutes, which form part of the intestinal microbiota of most mammals and birds, and have emerged as important nosocomial and community-acquired pathogens (Jung *et al.*, 2007). The isolation and identification of Treponeme, *Corynebacterium* and Firmicutes is in agreement with previous works that employed molecular identification of microorganisms from BDD lesions (Zinicola *et al.*, 2015).

The culture derived from sample 9MC (Tab.1) had best hits with *Cupriavidus gilardii*, a Gram-negative, motile rod that occurs in the environment, soil and human clinical specimens (Vandame and Coeny, 2004). *C. gilardii* has been isolated from human clinical samples that include respiratory tract infection (cystic fibrosis patients, Lipuma, 2010).

It was quite surprising to have isolated and identified *Leptospira* species derived from biopsies of BDD lesions. As previously stated,

the sequences from samples A8T and 8MC had best hits with *L. broomi* and *L. fainei* and that from sample 7MC had best hits with *L. licerasiae* and *L. wolffii* (Tab.1). Therefore, in three out of seven cultures, isolated from different individual cows from different farms, we positively identified *Leptospira* that could belong to four distinct species: *L. broomi*, *L. fainei*, *L. licerasiae* or *L. wolffii*. We could not find in the literature any previous description of the association of this genus with BDD lesions.

Leptospirosis is a worldwide zoonotic disease caused by infections with pathogenic spirochetes of the genus *Leptospira* which affects wild and domestic animals and humans. Infected animals excrete the organism in their urine and infect other animals by direct exposure to the contaminated urine or through indirect contact via contaminated water or soil (Zuerner, 2010). Comparisons of 16S rRNA sequences and DNA-DNA reassociation studies distinguished the presence of three clades in the genus *Leptospira*, the first is of pathogenic *Leptospira*, the second of intermediate pathogenic species and the third is of nonpathogenic species (Levett, 2015). There are five species with intermediate pathogenicity: *L. fainei*, *L. inadai*, *L. broomi*, *L. licerasiae* and *L. wolffii* (Levett, 2015). It is interesting to mention that all *Leptospira* isolated in the



present work (*L. broomii*, *L. fainei*, *L. licerasiae* or *L. wolffii*) belong to the intermediate pathogenic clade (Zuerner, 2010). These intermediate pathogenic species are found to cause human and animal disease. For instance, *L. broomii* was isolated from the blood, cerebrospinal fluid and urine of human patients with leptospirosis in Denmark and France (Levett *et al.*, 2006). *L. fainei* was previously isolated from cultures of uteri and kidneys of sows from two geographically distant pig herds in Australia (Perolat *et al.*, 1998). *L. fainei* was successively cultured from two human patients with Weil's disease and antibody titers have been found in sera from cattle in Australia, France and Indonesia (Chappel *et al.*, 1996; Petersen *et al.*, 2001).

*L. wolffii* was identified as a potential pathogenic species, detected in infected humans, in sheep sera and dogs urine and can be potentially pathogenic to human and probably animal hosts (Zakeri *et al.*, 2010); and *L. licerasiae* causes mild disease in humans and has been isolated from peridomestic and wild rodents and marsupials in Peruvian Amazon (Matthias *et al.*, 2008; Ricaldi *et al.*, 2012) and from environmental water (Saito *et al.*, 2013).

Krull *et al.* (2015) showed that early BDD lesions had minimal populations of *Treponema* spp. that grow significantly in more advanced and active lesion stages. In contrast, early lesions were composed of much more complex bacterial communities suggesting that it would have developed as a result of the concurrent presence of many bacterial species at the same time. The authors admitted that these findings fit the hypothesis that the treponemes represent an opportunistic organism that colonizes pre-existing lesions of the foot of dairy cattle. As the Treponeme species fail to have significant genetic capabilities to break down the skin barrier, they likely colonize the hospitable niche associated with skin lesions induced by other bacterial organisms or physical trauma to the foot (Krull *et al.*, 2015). In contrast to *Treponema* species that fail to have significant genetic capabilities to break down the skin barrier (Krull *et al.*, 2015), *L. interrogans*, the most common genotype in pathogenic *Leptospira* species, encodes a collagenase (*colA* gene), which was recently related to invasiveness and transmission of the spirochetes in cell monolayers and animals, being characterized as a

novel and crucial virulence factor (Kassegne *et al.*, 2014). Ricaldi *et al.* (2012) sequenced genomes of two strains of *L. licerasiae* and these had the highest average protein identity with *L. interrogans*. These observations suggest that *L. licerasiae* is more closely related to the pathogenic branch of infectious *Leptospira* than to the saprophyte *L. biflexa*.

Our results are in general agreement with evidences that point to the polymicrobial nature of the BDD. The unexpected finding of the isolation and identification of *Leptospira* of intermediate pathogenicity from deep BDD lesion biopsies indicates the need for further investigations in order to clarify whether, besides the treponeme, other spirochetes, as *Leptospira*, could be associated with BDD and even the probability of its participation in the pathogenesis of disease. Another interesting hypothesis would be that BDD skin lesions would allow the access of *Leptospira* of intermediate pathogenicity as a route to cause Leptospirosis in cattle, especially in tropical countries, clarifying whether the results of the present work could have any veterinary and medical significance.

## CONCLUSION

Multiple bacterial species are associated with BDD lesions. Here we report the isolation and identification of *Treponema pedis*, *Corynebacterium appendicis*, *Enterococcus casseliflavus/ gallinarum*, *Cupriavidus gillardi* and *Leptospira* that could belong to four distinct species: *L. broomii*, *L. fainei*, *L. licerasiae* or *L. wolffii*. Finding *Leptospira* of intermediate pathogenicity from deep BDD lesion biopsies indicates the need for further investigations in order to clarify whether, besides the treponeme, other spirochetes, as *Leptospira*, could be associated with BDD and even the probability of its participation in the pathogenesis of disease.

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