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# Detection of *Treponema* phylotypes from digital dermatitis lesions and effect of different phylotypes on lesion size

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## Abstract

Bovine digital dermatitis (BDD) is a contagious infectious disease which causes lameness in dairy cows. It has a multifactorial etiology which is not yet fully understood but *Treponema* spp. seem to play a significant role in development of BDD lesions. This study evaluated the presence of *Treponema* phylotypes commonly associated with BDD (*T. medium/T. vincentii, T. phagedenis* and *T. putidum/T. denticola)*, in four farms different areas in Iran. Single biopsies were taken from 113 Holstein cows with active BDD lesions (scored according to size) on the farms and polymerase chain reaction assays used to detect 16S rRNA nucleotide fragments of three BDD *Treponema* phylotype groups: "*T. medium/T. vincentii*", "*T. phagedenis*" and "*T. putidum/T. denticola*" (now *T. pedis*). Over 95.00% of samples were positive for at least one of phylotypes, with 89.00%, 91.00 %, and 66.00% of samples were positive for *T. putidum/T. denticola, T. phagedenis* and *T. medium/T. vincentii*, respectively. Out of the 113 samples, 60.00% were positive for all three phylotypes, the detection of *T. putidum/T. denticola* was positively associated with detection of both *T. phagedenis* and *T. medium/T. vincentii*. No association between lesion size and phylotypes identified was found but there were significant differences between farms in the proportion of each phylotypes identified. Further research is required to establish the factors influencing the proportions of individual phylotypes, especially at the farm level.



## Introduction

Bovine digital dermatitis (BDD) is an infectious disease of the skin of cattle which is probably the most important cause of infectious lameness in dairy cows. It was first described by Cheli and Mortellaro in the 1970s.[1] The BDD can result in high incidence of painful skin lesions, thereby significantly reduces animal welfare.[2] BDD also reduces profitability by decreasing milk production and reproductive performance and increasing culling rate, therefore, necessitates large preventative costs.[3,4]

High herd level prevalence of BDD (*e.g.*; 90.60% of dairy herds in the Netherlands, 70.00% in United Kingdom, 63.80% in New Zealand) makes the disease an important concern although the cow level prevalence has variation among different regions (i.e., 1.20% in New Zealand and 21.20% in the Netherlands).[5,6] Although BDD was first reported in Iran in 1991,[7] but no report of cow or herd level prevalence in Iran is available except for annual incidence of BDD was reported as 11.66% in a study of four large farms in Iran.[8]

The epidemiology and bacteriology of BDD has been studied all over the world.[9-13] Nevertheless, the precise etiology of BDD is still not completely clear. BDD appears to be a polymicrobial disease with multiple different species of bacteria isolated from or detected in BDD lesions, including *Bacterioides spp., Guggenheimella bovis, Campylobacter spp., Fusobacterium spp.* and *Peptococccus spp.* Spirochaetes specifically *Treponema* spp., seem to be the most critical bacteria in clinical BDD. However, difficulties in culture of *Treponema* culture and the relatively high percentage of bacteria in BDD lesions mean that culture-independent methods are more useful in studying the etiology of BDD.[10,11]

Forty-five species of Treponemes have been identified in BDD lesions, although most of these Treponemes belong to one of three phylotypes: *T. medium/T. vincentii*, *T. phagedenis* and *T. putidum/T. denticola* (*T. pedis*) that

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classified in different phylotypes showing about 2.00% difference from known species and more than 99.00% similarity to the members of the cluster.[10,13-16] It has shown that there is some evidence of geographical variation in distribution of *Treponema* phylotypes. *T. brennaborense* and *T. socranski* have not always been detected in all studies or detected in very low numbers of lesions.[10,17-20]

The *T. denticola, T. maltophilum, T. medium, T. putidum, T. phagedenis* and *T. paraluiscuniculi* were mostly detected in active lesions.[21] Development of the lesion is associated with the phylotypes of Treponemes and spirochetes are isolated from BDD lesions with different *in vivo* virulence and antigenic traits.[22,23] It was proved that *T. phagedenis* can affect wound repair and suppress bovine macrophages.[22] The *T. phagedenis* strains showed different pathogenic potentials by inoculating into a mouse abscess.[23]

Multiple species of Treponemes have been detected in BDD lesions, these Treponemes vary significantly in their pathogenic potential within and across species.[22,23] There is only limited evidence on the factors determining which Treponemes are present in BDD lesions. There is some evidence of geographical variation,[10,17-20] but the main factor seems to be the stage of lesion development.[13,21] BDD Lesions vary greatly in size[24,25] (as one aspect of lesion development) and its relationship with the *Treponema* species present has not been evaluated.

No previous polymerase chain reaction (PCR) detection of *Treponema* phylotypes has been collected in Iran, thus, the aim of this study was to detect three *Treponema* phylotypes (*T. medium/T. vincentii, T. phagedenis* and *T. putidum/T. denticola)* in four dairy farms and to evaluate the association between lesion size and the presence of different phylotypes.

## Materials and Methods

**Dairy farms and sample collection.** Single biopsies were taken from 113 Holstein cows with BDD from four farms in different geographical area of Iran. All farms were large commercial dairy farms (720 - 3,300 dairy cows) with free stall (three farms) and loose stall barns (farm 3) as the main housing systems. Farm one (3,300 dairy cows and average daily milk production of 33.00 L per cow daily, number of collected samples: 40, annual rain fall: 789 mm with 75.00% average humidity, and 3.08% BDD incidence rate, 36.5659° N, 53.0586° E) located in north, farm two (720 dairy cows; 35.00 L per cow daily, number of collected samples: 27, annual rain fall: 255 mm with 53.00% average humidity, and 4.86% BDD incidence rate,36.6416° N, 59.1214° E) and farm three (1,080 dairy cows; 39.00 L per cow daily, number of collected samples: 25, annual rain fall: 240 mm with 49.00% average humidity, and 1.52% BDD incidence rate, 36.2141° N, 58.7961° E) in north-east and farm four (900 dairy cows ; 37.00 L per cow daily, number of collected samples: 21, annual rain fall: 240 mm with 51.00% average humidity, and 4.50% BDD incidence rate, 35.7018° N, 59.8468° E), in the east of Iran. Hoof care programs (regular hoof trimming, locomotion scoring, hoof bathing, digital lesions diagnosis and treatment) were undertaken on all farms. Active digital dermatitis lesions were identified by a local veterinarian or professional hoof trimmer in hoof trimming chute, were scored according to its size (diameter of the lesion) and assigned into three score groups[26] as follow: Score zero: Without any lesion, Score one: Lesions less than or equal to 2.50 cm, and Score two: Lesions more than 2.50 cm in diameter. Five-millimeter punch biopsy was taken from score one and two lesions. Samples were rinsed with sterile saline, placed in phosphate buffered saline (PBS) and quickly transferred to laboratory, and frozen in – 80.00 ˚C till DNA extraction.

**DNA extraction.** Extraction of DNA was done using Geneall™ kit (GeneAll, Seoul, South Korea), according to the manufacturer's instructions. The concentration of DNA and the ratio of their absorbance at 260 and 280 nm were quantified by Nanodrop (Thermo Fisher, Waltham, USA). The extracted DNA was stored at – 80.00 ˚C until PCR testing.

**PCR.** The PCR assays for detecting three BDD *Treponema* phylotypes "*T. medium/T. vincentii*", "*T. phagedenis*" and "*T. putidum/T. denticola*" (now *T. pedis*) was performed using primers designed by Beacon Designer Software (Premier Biosoft, San Francisco, USA) based on 16S rRNA gene. The sequences of the forward and reverse primers were as follow, respectively: *T. putidum/T. denticola,* 5′-ACTTAATGCGTTTGCGTC-3′ and 5′-ACGCTGA TATACGAAGGTG-3′; *T. phagedenis*, 5′- GAAGAATAAGAGG ATGAGGG -3′ and 5′- AATAATTCCGAACAACGC -3′; *T. medium/T. vincentii*, 5′- AGCTTGCTTCTCTCCTAG -3′ and 5′- CCCTTATGAAGCACTGAG -3′. The predicted band size was 120 bp for all. Temperature cycling entailed 94.00 ˚C for 4 min was followed by 30 cycles of 94.00 ˚C for 30 sec, annealing for 30 sec at 45.00 ˚C for *T. medium/T. vincentii* primers, 30 sec at 94.00 ˚C for *T. phagedenis* primers, and 30 sec at 45.50 ˚C for *T. putidum/T. denticola* primers, an extension step at 72.00 ˚C for 30 sec and then a final elongation step at 72.00 ˚C for 5 min. The PCR reactions were performed in a final volume of 20.00 µL containing 17.00 µL of PCR premix (Bioneer, Daejeon, South Korea), 1.00 pmol of each primer and 20.00 ng of DNA template. Positive controls (samples with positive PCR and sequencing results in pilot tests using current study primers) and negative controls (deionized sterile water) were included in each analysis. Following PCR, 6.00 µL of the amplified products were loaded on a 1.30% agarose gel, and visualized by staining with ethidium bromide. These results were then compared to DNA marker (50 bp ladders; SMOBIO, Hsinchu City, Taiwan).

**Sequencing.** To confirm the specificity of PCR positive samples two PCR positive products for each phylotype in a volume of 50.00 µL were sent for sequencing (Bioneer).

**Statistical analysis.** Association between the detection of phylotypes within individual lesions were assessed by measuring the phi correlation coefficient. The effect of farm and lesion size on the proportion of lesions with each phylotype was assessed using chi-square/fisher exact test (version 26.0; IBM Corp., Armonk, USA).

## Results

Across the 113 hoof skin samples, all three *Treponema* phylotypes were detected using the PCR tests (Fig. 1). Over 95.00% of samples were PCR positive for at least one *Treponema* phylotype. Sequencing confirmed the presence of the 16S rRNA nucleotide fragments in all six positive samples submitted to Bioneer.

The percentage of lesions for T. *putidum/T. denticola*, *T. phagedenis* and *T. medium/T. vincentii* were 89.00%, 91.00% and 66.00 %, respectively. Three phylotypes were detected in 60.00% of biopsies. The highest rate of concurrent infection with two phylotypes in one lesion was 96.00% for *T. putidum/T. denticola* and *T. phagedenis*. Lesions positive for *T. putidum/T. denticola* were more likely to be positive for *T. phagedenis,* ($p < 0.001$) and *T. medium/T. vincentii,* compared to negative lesions ($\varphi$ = 0.50 and 0.24, respectively). In contrast, there appeared to be no relationship between the detection of *T. phagedenis* and *T. medium/T.* (Chi square, $p > 0.05$, $\varphi = 0.10$), (Fig. 2).

Totally, concurrent infection in two or more phylotypes of Treponemes in one lesion was not related to findings of another phylotypes of Treponemes.

The distribution of different Treponemes in farms is shown in Table 1. No effect of farm on the proportion of lesions with positive PCR results for *T. putidum/T. denticola* and *T. phagedenis* was observed ($p > 0.05$). In contrast, the proportion of lesions in which *T. medium/T. vincentii* phylotypes were detected was lower overall and much lower on farm 3 compared to the other three farms. There was a significant effect of farm in the proportion of lesions in which *T. medium/T. vincentii* phylotypes were detected ($p < 0.001$), but this farm effect was not significant if data from farm 3 were excluded ($p > 0.05$).

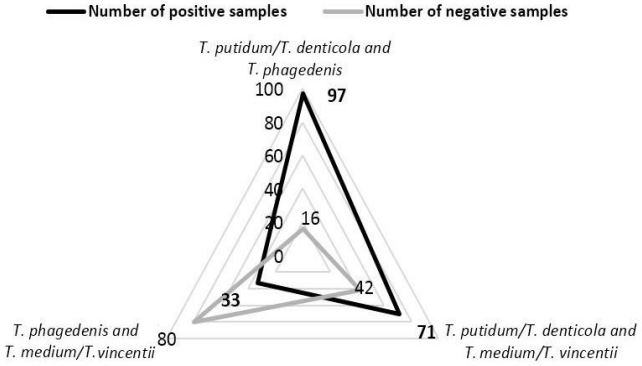


**Fig. 2.** Relation of different *Treponema* phylotypes in lesions.

Over all the farms, 64.00% of lesions were recorded as score 1. In farm 1, 28 samples out of 40 were in score 1 category. The number of score 1 samples in farm 2, 3 and 4 were 16 out of 27, 14 out of 25 and 14 out of 21, respectively. No farm effect on proportion of lesion within each score category was recorded ($p > 0.05$). The effect of lesion scores on detection of the three phylotype groups is summarized in Table 2. No association was found between lesion size and the detection rate of an individual phylotype ($p > 0.05$), nor was there any effect of size on likelihood of finding a phylotype combination.

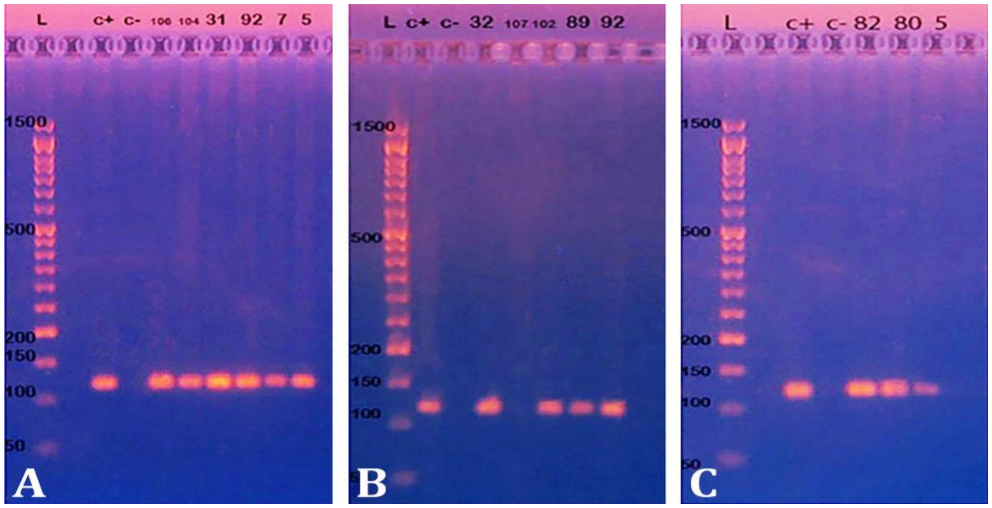


**Fig. 1.** The PCR amplification products for detecting *Treponema* phylotypes following electrophoresis. **A)** PCR products of *T. phagedenis* amplification: L: 50 bp DNA ladder, C+: *T. phagedenis* positive control, C-: *T. phagedenis* negative control, 106: *T. phagedenis* positive sample, 104, 31, 92, 7, 5: *T. phagedenis* positive samples; **B)** PCR products of *T. medium/T. vincentii* amplification: L: 50 bp DNA ladder, C+: *T. medium/T. vincentii* positive control, C-: *T. medium/T. vincentii* negative control, 32, 107, 102, 89, 92: *T. medium/T. vincentii* positive samples; and **C)** PCR products of *T. putidum/T. denticola* amplification: L: 50 bp DNA ladder, C+: *T. putidum/T. denticola* positive control, C-: *T. putidum/T. denticola* negative control. 82, 80, 5: *T. putidum/T. denticola* positive samples.

**Table 1.** Percentage of positive sample for Treponemes in different farms.

| Phylotype | Farm 1 | Farm 2 | Farm 3 | Farm 4 |
|---|---|---|---|---|
| ***T. putidum/T. denticola*** | 34.84 | 25.93 | 22.88 | 20.95 |
| ***T. phagedenis*** | 36.90 | 25.93 | 23.92 | 19.91 |
| ***T. medium/T. vincentii*** | 31.77* | 22.81* | 4.16* | 18.86* |

* indicates significant difference between farms ($p < 0.05$).

**Table 2.** Distribution of different phylotypes in different lesion scores.

| Phylotype | Total number of positive samples | Score 1, No. (%) | Score 2, No. (%) |
|---|---|---|---|
| ***T. putidum/T. denticola*** | 101 | 63(63.00) | 38(38.00) |
| ***T. phagedenis*** | 103 | 67(65.00) | 36(35.00) |
| ***T. medium/T. vincentii*** | 75 | 47(63.00) | 28(38.00) |
| ***T. putidum/T. denticola + T. phagedenis*** | 97 | 62(64.00) | 35(37.00) |
| ***T. putidum/T. denticola + T. medium/T. vincentii*** | 71 | 43(61.00) | 28(39.00) |
| ***T. phagedenis + T. medium/T. vincentii*** | 70 | 44(63.00) | 26(37.00) |
| **Concurrent infection of three phylotypes** | 68 | 42(62.00) | 26(38.00) |

## Discussion

The three phylotype groups of Treponemes most commonly identified in previous studies of BDD outside of Iran Evans *et al*., Klitgaard *et al*., Nordhoff *et al*., Yano *et al*. were detected using PCR in the current study method as well as different parts of the world.[10,11,18,19] *T. putidum/T. denticola*, *T. phagedenis* and *T. medium/T. vincentii* were detected in 89.00%, 91.00% and 66.00% of samples, respectively. Our findings showed that *T. phagedenis* was the most prevalent phylotype hat was consistent with previous studies.[10,18,27,28] Nordhoff *et al.* detected *Treponema* phylotype DDKL-4 (*T. phagedenis)* in 100% of acute lesions using DNA-DNA dot blot hybridization and FISH (Fluorescence in Situ Hybridization) analysis.[18] Evans *et al.* using the PCR technology used in the present study found *T. phagedenis* in 98.00% of BDD lesions.[27]

For the other two phylotype groups, Evans *et al*. reported a higher proportion of *T. medium/T. vincentii and* a *lower* proportions of *T. putidum/T. denticola* compared to our findings (i.e., 96.00% and 76.50% of the lesions).[27] Yano *et al.* reported that *T. denticola* and *T. phagedenis* were dominant phylogroups and *T. medium/T. vincentii as* less prevalent in BDD lesions, indicating that there was more variability in detection of these phylotypes compared to *T. phagadenis.*[10]

In the current study, most lesions had more than one *Treponema* phylotype present, consistent with the findings of previous studies and the hypothesis that there may be synergism between different phylotypes in BDD lesions.[18,19,27,29] For example, Klitgaard *et al.*, found two phylotypes of PT1 and PT6 (*T. phagedenis* ) in 90.00% of lesions,[19] while Nascimento *et al.* detected *T. phagedenis* and *T. medium/T. vincentii* in 95.00% and *T. phagedenis* and *T. putidum/T. denticola* in 86.00% of samples.[29] In this study ~60.00% of lesions had all three phylotype groups which were moderately lower than in previous studies. Klitgaard *et al*.,[19] Evans *et al.* and Nascimento *et al.* reported three phylotypes found in 78.00%, 74.50% and 82.00% of lesions, respectively.[27,29]

We hypothesized that different phylotypes in a lesion may affect lesion size, however, we found out there was no association between size of lesion and detection of individual phylotypes or their combinations. In contrats, Klitgaard *et al*.[19] reported that lesions with the highest pathology score (modified from Read and Walker)[9] had the lowest frequency of detection of all three phylotypes.

The differences in detection of the three phylotypes may be partly due to differences in sensitivities of three PCR tests. Döpfer *et al.* reported that their *T. medium* isolate did not have the same affinity for the primers and probe developed for the study as the other Treponemes were detected, however, further testing is required to confirm.[30]

This study was the first study using PCR to detect Treponemes in dairy cattle with BDD. All three phylotypes which have been commonly associated with BDD in other countries were identified on the four farms included in the present study, with *T. phagedenis* being the most commonly identified isolate that was consistent with other reports. For the least commonly isolated phylotype, *T. medium/T. vincentii*, there was a significant farm effect on detection rate with a very low isolation rate on one farm. Further research is required to establish the factors affecting the detection rates of *Treponema*.

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## Conflict of interest

The authors declare that there is no conflict of interest.

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