Molecular Detection of *Treponema* species Organisms in Foremilk and Udder Cleft Skin of Dairy Cows with Digital Dermatitis

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Abstract

Identification of reservoirs and transmission routes of digital dermatitis (DD) associated *Treponema* spp. considered an effective means for controlling DD infection in dairy cows. The objective of this study is to identify and characterize the potential reservoir niches for DD-associated *Treponema* spp. from healthy udder cleft skin and foremilk in lactating dairy cows. A large dairy farm was visited weekly from March to July 2015. Clinical investigation revealed that a total of 25 lame cows had DD lesions located at the plantar aspect of the interdigital cleft. A total of 75 samples, three per cow, were collected including deep swabs from DD lesions (*n*=25), non-aseptically collected foremilk samples (*n*=25) and skin swabs from udder cleft (*n*=25). *Treponema* spp. were identified using nested PCR assays and confirmed by DNA sequencing. Results revealed that *Treponema phagedenis* (*T. Phagedenis*)-like was the most identified species in the foremilk 40% (10/25), in comparison to DD lesions and udder cleft skin samples with 32% (8/25) and 20% (5/25), respectively. On the other hand, *Treponema pedis* (*T. Pedis*) was the most identified species in the udder cleft skin 80% (20/25), in comparison to DD lesions and foremilk samples with 68% (17/25) and 60% (15/25), respectively. None of the examined samples were identified by PCR as containing DNA from *Treponema medium* (*T. Medium*) or *Treponema vincentii* (*T. vincentii*)-like. To the best of our knowledge, this is the first report for detection of *T. phagedenis*-like and *T. pedis* from healthy skin of udder cleft and foremilk samples. Detection of DD *Treponema* spp. from udder cleft skin and foremilk samples indicates that these sites could be potential reservoirs for spirochetes involved in DD. Udder cleft skin and foremilk may have a role in transmission routes of DD *Treponema* in dairy farms.

Keywords: Dairy cows; Digital dermatitis; *Treponema*; reservoirs; Udder cleft

Introduction

Digital dermatitis (DD) is an important ulcerative infectious disease affecting bovine foot worldwide, leading to an epidemic lameness and economic losses in dairy cattle (Refaai et al. 2013; Evans et al. 2016). Digital dermatitis is highly contagious and may affect over 80% of cows within a herd (Holzhauer et al. 2006). *Treponema* spp., the DD causative agent, are typically anaerobic spirochetes, fastidious, highly motile, spiral microorganisms. *Treponemes* may be found in the oral cavity, digestive tract, and genital areas of humans, animals, and insects (Smirbert et al. 1984; Lilburn et al. 1999; Collighan et al. 2000; Evans et al. 2012). Previous investigations support the involvement of spirochetes of the genus *Treponema* in the DD pathogenesis (Nordhoff et al. 2008; Yano et al. 2010; Brandt et al. 2011; Evans et al. 2012; Clegg et al. 2016a).

Identifying the infection reservoirs and transmission routes of DD *Treponema* is crucial to minimize the spreading of infections and controlling the DD occurrence (Orsel et al. 2018). Although *Treponema* spirochetes are highly associated with DD lesions, it is unclear whether foot tissues are the primary infection reservoir or if there are other DD *treponema* niches in the cow or the dairy farm environment. The reservoirs for *Treponema* have not yet been fully identified and further studies are necessary for understanding their transmission and subsequently, the epidemiology of bovine DD (Evans et al. 2009, 2012). Recent studies had reported that DD associated *Treponema* spp. are correlated with other lesions on cattle skin, including several non-healing foot lesions, hock lesions, udder cleft dermatitis and ischaemic teat necrosis (Evans et al. 2010, 2011; Clegg et al. 2016a, b). Further reservoirs and hosts for *Treponema* have been also documented including non-pedal bovine regions such as oral cavity, bovine rectum, bovine gastrointestinal tract contents, and slurry, confirming the presence of different hosts and environmental reservoirs for *Treponema* other than the foot tissue or DD lesions. (Edwards et al. 2004; Evans et al. 2012; Klitgaard et al. 2017).

No available microbiological studies have previously been carried out to identify *Treponema* either from milk samples or healthy skin of udder cleft of dairy cows. Digital dermatitis associated *Treponema* was detected deep within lesions in bovine ulcerative mammary dermatitis cases (Evans et al. 2010). More recently, Clegg et al. (2016b) reported a high association between the presence of DD-associated *Treponema* and incidence of ischaemic teat necrosis. Association between udder lesions, in the form of ischaemic teat necrosis and udder cleft dermatitis, and DD-associated *Treponema* in dairy cows was documented (Stamm and Trott 2006; Stamm et al. 2009; Evans et al. 2010). However, to the best of our knowledge, there is no available literature that characterizes the DD-associated *Treponema* from extra-and intramammary sites as potential reservoirs. The objective of this study is to identify and characterize the potential reservoir niches for DD-associated *Treponema* spp. from healthy udder cleft skin and foremilk in lactating dairy cows using nested PCR assays and DNA sequencing. The findings of this study would boost our understanding and knowledge for the transmission of DD-associated *Treponema* spp. in dairy farms and that indeed will enhance the current control strategies for minimizing the contagious spread of DD in dairy cattle populations.

Materials and Methods

Study population and animals' selection

A large dairy herd comprised of 300 Friesian cows with a conventional milking system located in Sharkia province, Egypt was included in the present study (Refaai et al. 2017). The dairy herd has a frequent occurrence of lameness despite the routine hygienic program for foot health. The farm was visited weekly from March to July 2015. Dairy cows were selected based on persistent lameness and lesion of DD. Cows were housed on an earthen floor and were kept under the same conditions for the whole study period. Before each visit, the dairy farmers isolated cows that had abnormal gait after exit from milking parlor for inspection and further examination of the cows' feet. Affected limb/s was thoroughly examined in a claw-trimming box for the detection of claw disorders. Out of

all examined cows, 25 cows with typical characteristic lesions of DD were included in this study. Lesions of DD were represented as highly painful, erosive ulcerations of more than two cm in diameter located mainly at the plantar aspect of the rear feet, affecting the skin adjacent to the interdigital cleft (Döpfer et al. 1997; Schroeder et al. 2003), supplementary file 1.

Sample collection

Three samples types including DD lesions swabs, udder cleft skin swab, and non-aseptic foremilk samples were collected from 25 lame cows for detections of the DD-associated *Treponema* spp. Briefly, after securing each animal, DD lesions were flushed with running water and sterile swabs were inserted deeply in the lesions and rolled roughly until it became saturated then inserted into sterile tubes containing 1mL normal saline. Another sterile swab was used for sample collection from the apparently healthy skin of the udder cleft. The swabs were rolled and passed several times across the udder cleft then inserted into sterile tubes containing 1 mL normal saline. Composite foremilk samples from all functional quarters of each cow were collected non-aseptically in sterile clean tubes. The milk samples collected without scrubbing the teat end with 70% ethanol or discarding the first squirts of milk. All samples were transported on ice at 4° C to the laboratory where they were kept frozen at - 20° C for subsequent investigations.

DNA extraction and nested PCR assay

DNA was extracted from all collected samples using Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The yield and quality of DNAs were assessed by Q5000 UV-Vis spectrophotometer (Quawell Technology, Inc, USA). Purified DNAs were stored at -20° C until usage. A nested PCR was used to detect and classify *Treponema* spp. The first PCR was conducted with a universal spirochetes' primers (Table 1) to amplify 1526 bp fragment of 16S rRNA gene in a 25µL total volume containing 12.5µl of HotStarTaq Master Mix (Qiagen), 1µL of each primer (10 µM), 3µL of

genomic DNA and 7.5 µL deionized water. Amplification was carried out in Mastercycler X50 thermal cycler with an initial denaturation at 94°C for 5 min, followed by 37 cycles of denaturation at 94°C for 30 sec, annealing at 42°C for 30 sec, and extension at 72°C for 75 sec, with a final extension at 72°C for 10 min. The amplified fragment was separated in a 1.5% agarose gel and imagined under UV light in a gel documentation system (Bio Doc Analyse, Biometra, Germany). All positive samples in the first PCR exposed to a second reaction with three different specific primers for each *Treponema* type and 1 µl of the first PCR amplicon as a template. The second PCR condition was similar to the initial PCR except for annealing temperature which was differed from primer to another (Table 1).

Sequencing of amplified PCR products

The PCR products of nine representative samples from each predicted size were confirmed by sequencing. The PCR products were purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA) as per manufacturer's instructions. The purified amplicons were sequenced by Sanger sequencing method using the same forward and reverse primers as used in PCR. The obtained sequences were trimmed and aligned using "Sequencher 5.1" software followed by BLAST analysis in the GenBank database. The alignment of compatible nucleotide sequences was performed by using the Clustal W option in MEGAX (Molecular Evolutionary Genetic Analysis) software. A phylogenetic tree of aligned sequences was constructed by choosing the best fit Maximum Likelihood model in MEGAX based on lowest BIC score (Bayesian Information Criterion). The evolutionary distances were computed using the Kimura 2-parameter (Kumar et al. 2018).

Results

DD-associated *Treponema* spp. were identified using nested PCR assay and confirmed by DNA sequencing in the swab samples collected from DD lesions, the skin of the udder cleft, and foremilk samples. Genomic DNA was amplified in the first PCR reaction using universal spirochetes primers

for 16S rRNA gene, the amplified products were1526 bp. Second PCR revealed two amplicon sizes at 421 and 586 that were confirmed to be *Treponema phagedenis* (*T. phagedenis*)-like and *Treponema pedis* (*T. pedis*), respectively, by sequencing of nine representative purified amplicons (supplementary file 1). The obtained sequences were submitted to the GenBank database under Accession numbers MK732466, MK732467, MK732468, and MK732469 for *T. phagedenis*-like and MK732461 , MK732462, MK732463, MK732464 and MK732465 for *T. pedis*. Results revealed that *T. phagedenis*-like was the most identified species in the foremilk samples with a percentage 40% (10/25) in comparison to the udder cleft skin (20%, 5/25) and DD lesions (32%, 8/25) swabs samples. On the other hand, *T. pedis* was the most identified species in the udder cleft skin samples (80%, 20/25) in comparison to the foremilk (60%, 15/25) and DD lesions (68%, 17/25) swabs samples, Table 2. None of the examined sample were identified as containing *Treponema medium* (*T. medium*) or *Treponema vincentii* (*T. vincentii*)-like

The molecular analysis of the obtained sequences revealed that similarity among the T. phagedenis-like isolates was 100% while similarity among *T. pedis* isolates was 98-99%. The similarity between both types was 93%. That's why two isolates of the sequenced *T. pedis* were located in different clade in phylogenetic tree while all isolates of the sequenced *T. phagedenis*-like align in the same clade (Figure 1).

Discussion

DD-associated *Treponema* including *T. pedis, T. medium, T. phagedenis, and T. refringens* are the most abundant in the dairy herds (Moreira et al. 2018). However, *T. pedis* and *T. phagedenis* like were the only detected spp. among our samples. Identification of the potential reservoirs for DD *Treponema* was the main concern in many previous studies (Evans et al. 2012, 2016; Klitgaard et al. 2017). Numerous number of reservoirs for *Treponema* spp. were identified and reported from different hosts including bovine (Evans et al. 2012; Nascimento et al. 2015; Clegg et al. 2016a, b), ovine (Sayers et al. 2009; Duncan et al. 2014; Sullivan et al. 2015a, b; Crosby-Durrani et al. 2016),

177 pigs (Svartström et al. 2013; Karlsson et al. 2013), horse (Moe et al. 2010; Sykora and Brandt,
178 2015) and wild animals (Clegg et al. 2015). Continuous looking for new DD-associated *Treponema*
179 reservoirs is in need due to it is ability to expand not only their host range but also their tissue
180 specificity (Clegg et al. 2016a). This study showed that DD-associated *Treponema*, *T. phagedenis*-
181 like, and *T. pedis* are detected in the DD lesion, healthy udder cleft skin and foremilk samples of
182 dairy cattle. Thus, these sites can pose potential reservoirs for *Treponema* spp. and worrying routes
183 for the transmission of DD between animals in the dairy herds. Therefore, they may interfere with
184 the prevention and control program of DD in dairy farms.
185
186 There is association between udder lesions and DD-associated *Treponema* in dairy cows.
187 *Treponema* can infect skin wounds on areas other than the foot such as hock skin lesions and
188 pressure sores (Clegg et al. 2016a, c). Several *Treponema* spp. present in the lesions of udder cleft
189 dermatitis were associated with those isolated from bovine DD (Stamm et al. 2009).
190 *Treponema* spp. was previously isolated from the lesions of bovine ulcerative mammary dermatitis
191 cases (Stamm et al. 2009; Evans et al. 2010). Moreover, a high association between the presence of
192 DD-associated *Treponema* and incidence of ischaemic teat necrosis was confirmed (Clegg et al.
193 2016b). Based on 16S rDNA sequence, *T. phagedenis*-like was confirmed in Papillomatous digital
194 dermatitis (PDD) cases (Stamm and Trott 2006). Spirochetes also identified in samples from udder
195 cleft dermatitis lesions (Beattie and Taylor, 2000; Keil et al. 2002; Read et al. 2003; Evans et al.
196 2010).
197
198 However, other studies found no association between *Treponema* spp. detection in the udder
199 cleft dermatitis and presence of DD lesions (Warnick et al. 2002; Persson Waller et al. 2014). A
200 high percent of *Treponema* spp. was detected in healthy bovine foot tissues, lesion-free forefeet and
201 healthy hind leg tissue above the DD lesion. It was also detected in different sites other than feet,
202 including the oral cavity, rumen and recto-anal junction (Strub et al. 2007; Evans et al. 2012). Our

203 results revealed that the prevalence of *T. phagedenis*-like from DD lesions and healthy udder cleft
204 skin was high, indicating the important role of udder cleft skin as a reservoir for DD-associated
205 *Treponema* and support the idea of absence association between *Treponema* detection and presence
206 of lesion.
207
208 On the other hand, DD *Treponema* was identified in different body fluids and excretions in
209 dairy cows e.g., rumenal fluid, slurry and cow feces (Klitgaard et al. 2014; Nascimento et al. 2015).
210 Frössling et al. (2018) detected the *T. phagedenis*–like antibodies in serum and bulk milk from cows
211 with and without DD. In consistency, *Treponema* spp. was detected in our collected foremilk
212 samples that also could be a significant reservoir for DD *Treponema*. No previous studies were
213 detected *Treponema* in milk. Moreover, *Treponema* has never been considered or reported as a
214 mastitis causing pathogen or even as a normal flora of milk or intramammary tissues (National
215 Mastitis Council 1999).
216
217 Identification of DD *Treponema* in the healthy skin of udder cleft and foremilk samples may
218 be due to (i) contamination from DD lesions which disseminates *Treponema* continuously (ii)
219 colonization of the microorganism. The contamination hypothesis supported with the fact that DD
220 *Treponema* may be detected on the skin surface near active lesions due to it is ability to motile and
221 migrate over the animal body (Clegg et al. 2016a). Also, It could be detected in tissues distant to the
222 site of infection (Sell et al. 1980). *Treponema* spp. may use skin as a mode of transmission and
223 migrate using swarming motility (Clegg et al. 2016c). Environmental teat contamination may
224 happen during animal recumbency on contaminated ground. This also supported with the fact that
225 all 25 selected cows in this study were having DD lesions that make it a plausible and logic source
226 of contamination for other body parts. The milk samples were collected without discarding the first
227 milk streams (foremilk) according to recommendations of National Mastitis Council (1999). The

milk may expose to post-milking contamination while teat canal remains open for a few hours after the end of milking increasing chance for bacteria penetration (Tyler et al. 1997; Strapák et al. 2017).

Colonization hypothesis is supported by the ability of *Treponema* spp. to colonize in different environments based on the availability of essential factors for growth and proliferation (Wolgemuth et al. 2006). It has the ability to colonize in different tissues (Clegg et al. 2016c). Evans et al. (2012) identified the DD *Treponema* in two non-pedal bovine regions, the oral cavity (14%) and the rectum (15%). Further, *Treponema* other than those responsible for bovine DD can colonize in rumenal fluid and in the healthy interdigital cleft, and commonly associated with the healthy horn of the foot (Paster and Canale-Parola 1982; Evans et al. 2009, 2011; Nascimento et al. 2015). DD and non-DD *Treponema* spp. may be considered natural flora in a dairy farm environment and this could explain why they are commonly present on healthy hoof and foot skin. Therefore, future research is necessary for distinguishing between DD and non-DD *Treponema spp*.

Conclusion

DD *Treponema*, *T. phagedenis*-like and *T. pedis,* can be detected in healthy skin of udder cleft and foremilk samples suggesting that these niches are potential reservoirs for spirochetes involved in DD. Udder cleft skin and milk may have a role in transmission routes of DD *Treponema* in dairy farms.

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Compliance with ethical standards

Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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415 **Table 1.** Primers sequences used for the PCR assay of 75 samples from 25 Egyptian dairy cows for identification of *Treponema* spp.

Target	Sequence	Target region	Size (bp)	Tn	Reference
Universal (16SrRNA)	F:5-AGAGTTTGATCCTGG-3 R:5TACCTTGTTACGACTT-3	7–26 1491–1506	1,526	42	Rurangirwa et al. (1999)
Treponema medium/ Treponema vincentii-like	F:5-GAATGCTCATCTGATGACGGTAATCGACG-3 R:5-CCGGCCTTATCTAAGACCTTCTACTAG -3	465-493 994- 1011	546	55	KT192159.1 KT192148.1
Treponema phagedenis-like	F:5-GAAATACTCAAGCTTAACTTGAGAATTGC-3 R:5-CTACGCTACCATATCTCTATAATATTGC-3	644-672 1038-1065	421	50	M57739.1
Treponema pedis	F:5-GGAGATGAGGGAATGCGTCTTCGATG-3 R:5-CAAGAGTCGTATTGCTACGCTGATATATC-3	407-432 993-965	586	55	EF061267.1

416 F: forwad; R: reverse; bp: base pair; Tn: annealing temperature

417

418 **Table 2.** Prevalence of digital dermatitis associated *Treponema* spp identified by nested PCR assay

419 from digital dermatitis (DD) lesions, udder skin and foremilk of 25 Egyptian dairy cows.

Sample ID	*Treponema phagedenis*-like			*Treponema pedis*		
	Udder cleft (%)	DD lesion (%)	Milk (%)	Udder cleft (%)	DD lesion (%)	Milk (%)
1	+	+	+	-	-	-
2	-	-	+	+	+	-
3	-	-	+	+	+	-
4	+	+	+	-	-	-
5	-	-	-	+	+	+
6	+	-	-	-	+	+
7	-	+	-	+	-	+
8	-	+	+	+	-	-
9	-	+	+	+	-	-
10	-	-	+	+	+	-
11	-	-	-	+	+	+
12	-	-	-	+	+	+
13	+	-	-	-	+	+
14	-	-	-	+	+	+
15	-	-	-	+	+	+
16	-	-	-	+	+	+
17	-	-	-	+	+	+
18	-	-	+	+	+	-
19	-	+	+	+	-	-
20	-	+	-	+	-	+
21	-	-	-	+	+	+
22	-	-	-	+	+	+
23	+	+	+	-	-	-
24	-	-	-	+	+	+
25	-	-	-	+	+	+
Total	5(20)	8(32)	10(40)	20(80)	17(68)	15(60)

420

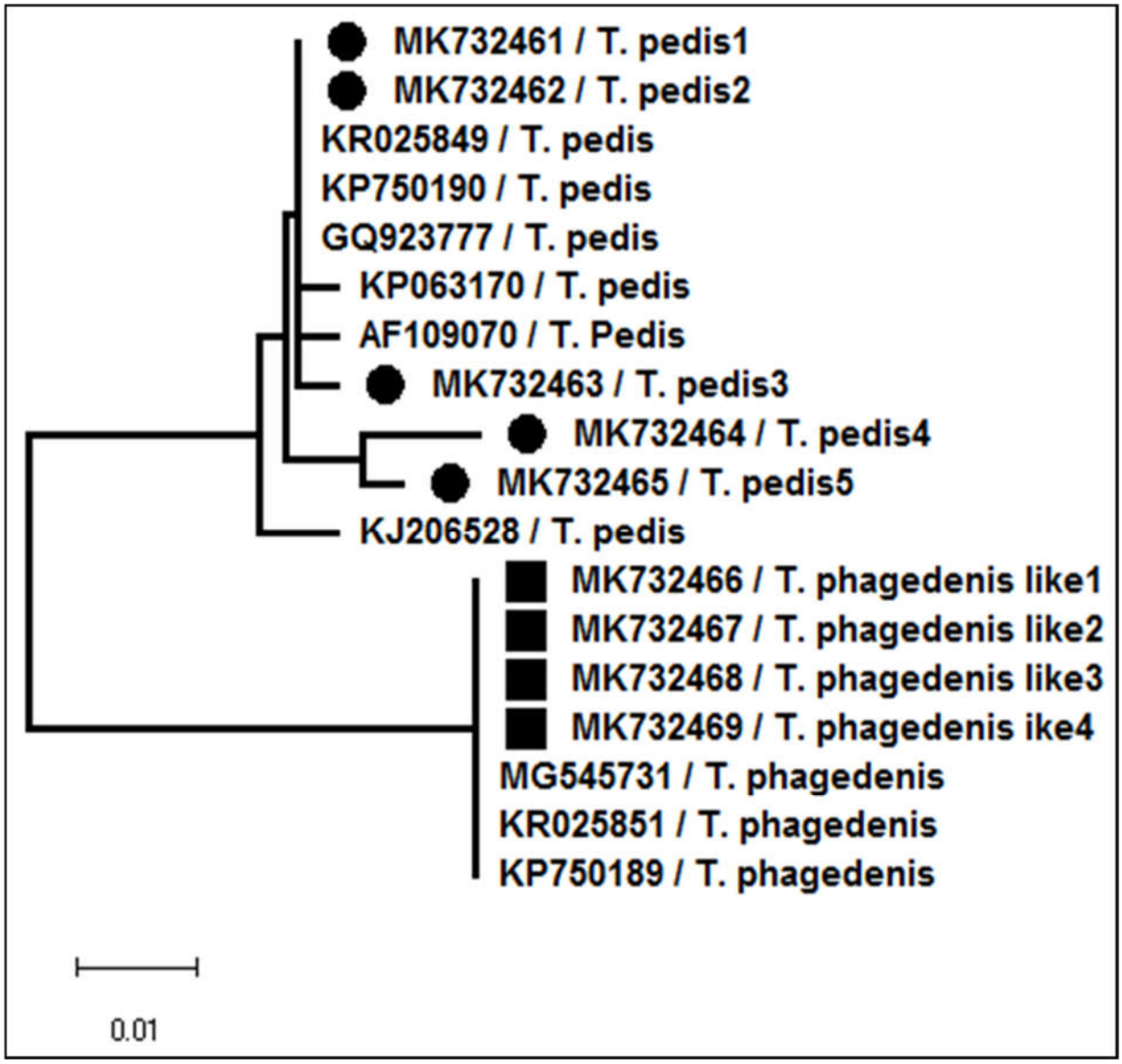

Figure 1. Phylogenetic analysis based on 300 nucleotides of the *Treponema* 16S rRNA.The tree shows relationship between different *Treponema* types using Maximum Likelihood method with Kimura 2 parameter mode.