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Molecular Isolation of Treponema spp from Ovine Footrot Lesions, Finding Evidence for Contagious Ovine Digital Dermatitis

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ABSTRACT

Contagious ovine digital dermatitis (CODD), a severe lameness-causing bacterial foot disease, significantly impacts the sheep industry's economy and welfare. Treponema species responsible for bovine digital dermatitis (BDD), including *Treponema medium*, *Treponema phagedenis*, and *Treponema pedis*, have been identified in CODD lesions. In this study, Treponema spp. was detected in 42 skin samples from 110 sheep that had footrot lesions (38%). The lesions were more presented on hindlimbs (55.5%). The Kurdish sheep breed exhibited the highest Treponema positivity rate with 47.8% (n = 20), while pure Afshari sheep and Afshari-Kurdish hybrids showed an equal prevalence of 26.2%. The prevalence of lesion scores 4 and 5 in the Treponema-positive group (33.3% and 40.5% respectively) was significantly higher compared to the Treponema-negative group (23.5% and 11.8% respectively) further underscores the potential role of Treponema infection in the progression of severity of the infection. The southern region of the Khorasan Razavi province exhibited a significantly higher prevalence of Treponema-positive cases (88.1%, n = 37) compared to the northern regions (7.1%, n = 3) and Mashhad (4.8%, n = 2). This is the first report of investigation of the possible presence of CODD in Iranian sheep flocks, which should be potentially considered to prevent negative impacts on sheep production, welfare, and antibiotic use (improper antibiotic usage) in the farms.

Introduction

Infectious foot diseases such as footrot and contagious ovine digital dermatitis (CODD) are the primary causes of lameness in sheep. CODD is a highly contagious bacterial foot disease characterised by severe lameness and has a major economic and welfare impact on the sheep industry.[1] Since the first case of the disease was discovered in the UK in 1997,[2] infectious causes of CODD were detected in up to approximately 50% of the UK sheep flocks.[3,4] While CODD has been a major concern in the UK, the disease has just recently been reported in other countries. It was first detected in Germany in 2020,[5] in Sweden in 2019,[6] and in Switzerland in 2022.[7]

The disease initially manifests as an inflammation of the coronary band, undermining the hoof horn capsule dorsally and abaxially, followed by progressive separation of the hoof capsule from the underlying tissue. In severe cases, the entire hoof horn may be detached, exposing the sensitive lamellae.[2,5,8,9] CODD can severely damage the soft tissues of the sheep's foot and cause bony changes in the distal phalanx.[8] Lesions typically occur on a single foot, but can affect more than one digit.[10] Lameness has been reported in more than 80% of the sheep with CODD foot lesions.[11]

The etiology of both footrot and CODD is bacterial. Previous microbiological studies have shown that CODD

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lesions are associated with Spirochetal bacteria, primarily *Treponema* genius.[12-14] Three Treponema species responsible for bovine digital dermatitis (BDD), including *Treponema medium*, *Treponema phagedenis* and *Treponema pedis*, have been found in CODD lesions.[14-16] Comparison of the prevalence of individual Treponema phylogroups in BDD in beef and dairy cattle and CODD in sheep showed little variation.[17] The BDD Treponemes are opportunistic infective agents, as previous evidence in cattle has demonstrated that they can invade other noninfective foot lesions, such as white line disease and sole ulcers, and manifest clinically as new serious infectious diseases which are extremely challenging to treat.[18]

There is evidence that other types of bacteria may also be important in the development of CODD. In addition to Treponema species, *Dichelobacter nodosus* (*D. Nodosus*) and *Fusobacterium necrophorum (F. necrophorum*), which are associated with footrot and digital dermatitis (DD), have been found in CODD lesions.[16,19] *D. nodosus* is the primary cause of ovine footrot, and *F. necrophorum* contributes to its severity as a secondary invader.[20-24]

Treponemes are anaerobic, highly fastidious bacteria that require especial conditions for isolation in culture media.[25] Due to the difficulties in Treponema culture and the high false-negative results, with regards to the high numbers of bacteria in foot lesions, polymerase chain reaction (PCR)-based methods are preferred for detecting these bacteria.[26] A PCR test can detect non-viable and dead cells, as well as live and difficult-to-culture cells.[27] It is recognized that bovine DD Treponeme-specific PCR assays can be used as differential diagnostic tools for CODD.[16,19] Previous studies have reported that Treponema DNA can frequently be amplified from samples of healthy bovine foot tissue when a genus-specific PCR test is used, a situation leading to increased false-positive outcomes. However, it is not observed when a DD Treponeme phylogroup PCR assay is used.[28,29]

Recently, CODD has received increased attention in sheep producing countries. This is due to the severity of the disease and the potential negative impact it can have on both individual sheep and the sheep industry as a whole. However, no previous PCR detection of Treponema phylotypes in sheep has been performed in Iran. The aim of this study was to detect three Treponema phylotypes (*T. medium/T. vincentii, T. phagedenis* and *T. putidum/T. denticola*) as the main causes of CODD, to investigate the possible contamination of CODD in sheep with footrot lesions.

Materials and Methods

Study Population and Sample Collection

The study was conducted on 110 sheep with apparent lameness due to hoof lesions, in the livestock market of Mashhad, Iran from October 2021 to February 2022. All sheep were carefully examined by an expertise veterinarian and sheep with digital lesions were selected and enrolled in the study. Date of sampling, breed, age, gender, geographical area, type of breeding and any possible systemic manifestation of disease (fever, mucosal hyperemia, anorexia and ruminal function) were recorded at the time of sampling. The lesions were scored according to the Swiss six-point scoring system for footrot,[30] with score zero being a healthy claw, score one for limited mild interdigital dermatitis, score two for more extensive interdigital dermatitis, score three for severe interdigital dermatitis and under-running of heel horn and sole, score four for further under-running spread to the walls of the hoof and score five for loss of the horn capsule. Following rinsing the foot lesions with sterile saline, a full thickness samples were taken from the affected feet using a five millimeters biopsy punch. Samples were placed in a plastic bag and cryotube, transferred rapidly to the laboratory and frozen at -20 °C until DNA extraction.

DNA Extraction and PCR Testing

The Geneall™ kit (GeneAll, Seoul, South Korea) was used for extraction of DNA, based on the manufacturer's instructions. The content of DNA and its absorbance at 260 and 280 nm were quantified by Nanodrop (Thermo Fisher, Waltham, USA). DNA was extracted and stored at –80 °C until PCR testing. Primers of a *Treponema* genius designed by Beacon Designer Software (Premier Biosoft, San Francisco, USA) based on 16S rRNA gene and samples were subjected to it. The sequences of the forward and reverse primers were TTACGTGCCAGCAGCCGCGGTAAC and GTCRYMGGCATTCCGCCWGAGTC, respectively. Temperature cycling consisted of 94.00 °C for 4 min followed by 30 cycles of 94 °C for 30 sec, 58 °C for 40 sec, and 72 °C for 40 sec, and then a final elongation step at 72 °C for 10 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

Two PCR-positive products in a volume of 50 µl were

sent for sequencing in order to verify the specificity of PCR-positive samples (Bioneer).

Statistical Analysis

Basic data were explained descriptively and associations between the presence of various factors and Treponema infection were assessed using Chi-square/Fisher exact test (version 26.0; IBM Corp., Armonk, USA).

Results

110 sheep with footrot lesions were examined and full details of the animals, geographical origin and lesion scores are recorded in Table 1. Skin samples from the interdigital space and lesions of the affected feet were investigated for Treponema by PCR. Treponema was isolated in 38.2% (n = 42) and Sequencing confirmed the presence of the 16S rRNA nucleotide fragments in all positive samples submitted to Bioneer.

Table 1. Characteristics of 110 footrot affected sheep, Lesion score, and the geographical area where the animal has lived (Treponema-positive [n = 42], Treponema-negative [n = 68]).

Description		Treponema	
		Positive	**Negative**
Gender (Number/percent)	Male	19/45.2	38/55.9
	Female	23/54.8	30/44.1
Affected limb (Number/percent)	Forelimb	17/40.5	32/47.1
	Hindlimb	25/59.5	36/52.9
Systemic symptoms (Number/percent)	Yes	0/0*	9/13.2*
	No	42/100*	59/86.8*
Breed (Number/percent)	Afshari	11/26.2	21/30.9
	Afshari- Kurdish	11/26.2	10/14.7
	Kurdish	20/47.8	37/54.5
Lesion score (Number/percent)	Score 2	3/7.1*	13/19.1*
	Score 3	8/19*	30/44.1*
	Score 4	14/33.3*	16/23.5*
	Score 5	17/40.5*	8/11.8*
Geographical area (Number/percent)	North of Mashhad	3/7.1*	28/41.2*
	South of Mashhad	37/88.1*	39/57.4*
	Mashhad	2/4.8*	1/1.5*

* indicates significant difference ($p > 0.05$)

Score 3 lesions were the most prevalent score among samples (n = 38, 34.5%), indicating severe interdigital dermatitis and underrunning of the heel-horn to the sole (Figure 1). This was followed by score 4 (n = 30, 27.3%) and score 5 (n = 25, 22.7%) with further underrunning extending to the hoof wall and loss of the horn capsule (Figures 2 and 3). The lowest frequency of lesions was observed in the score 2 category (n = 16, 14.5%).

The distribution of the lesion scores was different between the Treponema-positive and the Treponema-negative groups ($p < 0.05$). The most prevalent lesion score in Treponema-positives were score 5 with 40.5 % (n = 17), which was significantly higher than Treponema-negatives with 11.8 % (n = 8). Score 4 lesions were also higher in the Treponema-positive cases at 33.3% (n = 14) compared to 23.5% (n = 16) in the Treponema-negative cases. While the least prevalent lesion score in the Treponema-positive sheep was score 2 with 7.1% (n = 3), which was significantly lower than in the Treponema-negative group (19.1%, n = 13).

The number of males and females with footrot lesions were almost the same frequency as males comprising 51.8% (n = 57) and females accounting for 48.2% (n = 53) of Treponema positive lesions. However, a slightly higher non-significant prevalence of Treponema positivity among females (54.8%) compared to males (45.2%) were recorded ($p > 0.05$).

Lesions detected in 49 sheep (44.5%) in forelimbs and 61 sheep (55.5%) recorded with hindlimb involvement. Hindlimb lesions were more prevalent in both groups that was more prominent in Treponema-positive animals (59.9%) in comparison with Treponema-negative (52.9%).

The presence of systemic symptoms was noted in a few cases (n = 9, 8.2%), nonsympthomatic animals were almost all positive for Treponema that suggests that the disease is not related to the systemic infections.

The Kurdish breed constituted the majority of footrot affected sheep with 51.8% (n = 57) and Treponema-positive sheep with 47.8% (n = 20). Whereas, pure Afshari sheep and Afshari-Kurdish hybrids followed by 29.1% (n = 32) and 19.1% (n = 21) of footrot affected sheep, respectively, and showed an equal prevalence of 26.2% (n = 11 for each) for Treponema positivity. These results

suggest a possible genetic factor that affects susceptibility of the sheep to Treponema infection.

The number of footrot affected sheep was significantly higher in the southern regions of the Khorasan Razavi province with 69.1% (n = 76), followed by 28.2% (n = 31) and 2.7% (n = 3) in northern regions and suburban of Mashhad respectively. Additionally, the number of Troponema-positive sheep was also significantly higher in the southern regions (88.1%, n = 37) compared to the northern regions (7.1%, n = 3) and Mashhad (4.8%, n = 2) ($p < 0.05$).

Discussion

Presence of Treponema. spp in tissue samples of the footrot (like) lesions may be a clue for the possible presence of clinical outbreaks or cases of CODD in Iranian sheep flocks. CODD is clearly increasing in importance and geographical spread. The prevalence estimates have been reported ranging from 13% to 53% in the UK.[3,4] CODD has also been detected in Ireland, Germany and Sweden, as well as in digital dermatitis lesions in UK goats and American elk.[5,6,14,31,32]

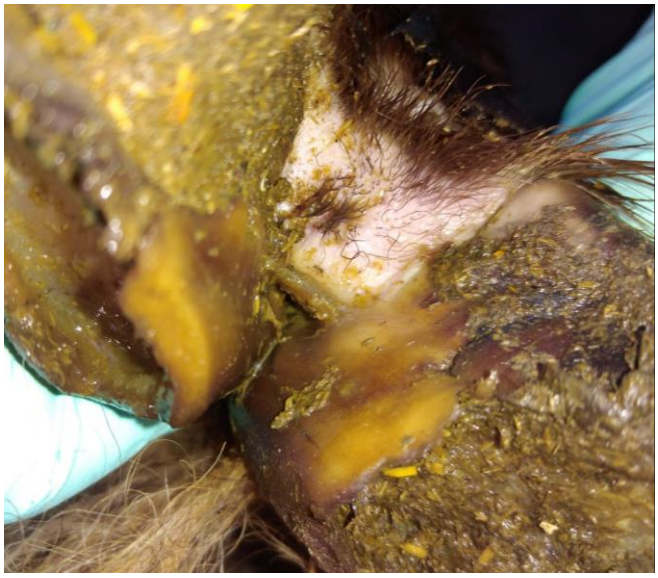

Figure 1. Footrot lesion score 3. Interdigital dermatitis and severe hair loss, underrunning of the hell towards the sole.

Figure 2. Footrot lesion score 4. Underrunning (including seperation) expands to the walls of the hoof, pododerma heavily affected.

Figure 3. Footrot lesion score 5. Removal of the hoof capsule, extended pododerma damages, inflamed interdigital space with hair loss.

It has become apparent that BDD Treponemes play a major causative role in CODD.[6,14,33] In a study one or more BDD-associated Treponema phylogroups were detected in 100% of CODD lesions. This confirms that the specific PCR assays are an effective differential diagnostic tool for CODD.[16]

In the present study, 42 of the 110 examined feet sheep (38.2%) (n = 42) of tested positive for the presence of the Treponema contamination. This finding highlights the significant prevalence of Treponema in sheep with footrot lesions. Most CODD lesions occurred after pre-existing ID and/or footrot lesions in the same digit[19]. Previous research suggests that CODD and footrot are two phases of the same infectious foot disease.[34]

In the present study, the distribution of affected limbs revealed a slightly higher incidence of hindlimb lesions (55.5%, n = 61) compared to forelimb (44.5%, n = 49). Additionally, the distribution of affected limbs differs among both Treponema-positive and -negative groups. The prevalence of lesions in the hindlimbs of Treponema-positive sheep was higher than in the forelimbs, as described in Table 1. Consistent with our findings, Duncan *et al*. (2011),[35] Duncan *et al*. (2012),[10] and Angell *et al*. (2015)[36] also found the majority of CODD lesions on hindlimbs. Although Angell *et al*. (2015) found the reverse distribution for footrot, with forelimbs more affected. This may be a result of possible specific predilection of Treponema for hindlimb tissues or a different mode of transmission in these cases.

Treponema infection rates have recently been reported in Swedish prevalence studies of footrot and/or CODD. In 2022, Rosander *et al*. detected Treponema spp.

in 65% of adult sheep and 82.8% of flocks.[37] Similarly, Treponema spp. was found at significant levels in 18 out of 20 sheep flocks in a previous field study in 2015.[20] Both investigations used swab sampling method and real-time PCR, which identified the entire Treponema genus without being able to differentiate between commensal and pathogenic species. In a previous study in the UK, Treponema DNA was detected in 70% of CODD lesions and 38% of healthy foot samples collected using a swab method.[27] This may be due to contamination with commensal treponemes found in the environment or in the GI tract of ruminants.[29] In another study by Rosander *et al*. in 2020, Treponema spp. was found in the 90.6% of the samples collected from slaughter lambs using the same methods.[38] However, Angell *et al*. found that the Spirochaetaceae family was more common in biopsy samples than in swab samples, suggesting that these bacteria are present in deeper tissues of the affected foot.[34] Sullivan et al. also reported the presence of one or more BDD-associated Treponema phylogroups in 100% of CODD lesion biopsy specimens in the UK in 2015, which is consistent with our findings. In addition, no BDD-associated Treponema was detected in biopsy samples from healthy sheep feet, while 68% were positive for general Treponema (Treponema genus-specific PCR).[16]

Although Treponema positivity in the present study was considerable (38.2%), it is much lower than previous results from prevalence studies performed in other countries. As reported by König, Nyman *et al*.[39] it is difficult to compare results from various countries because there are large differences in achieving methods (questionnaires, clinical examination, inspection at slaughter) and sampling (sampling method, severity of footrot, number of feet examined, age of animals).

Although CODD is distinct from footrot and ovine interdigital dermatitis, there appears to be a strong association between them.[3,36] Pre-existing interdigital dermatitis and/or footrot lesions may be a predisposing factor for most CODD lesions, as they injure the skin and allow access for Treponema spp.[19,36]

Breed differences in presence of footrot lesions were also evident in the present study. There were also differences in Treponema status between breeds. The higher prevalence of Treponema positivity among Kurdish sheep compared to other breeds suggests a potential genetic predisposition or environmental factor influencing susceptibility. Further genetic studies and environmental assessments are needed to investigate this hypothesis.

Lesion severity, as assessed by the assigned scores, provides valuable insights into the progression of footrot in the studied population. The significant higher prevalence of score 5 in the Treponema-positive group compared to the Treponema-negative group further underscores the potential role of Treponema infection in the progression of the lesion/infection severity. The significant lower prevalence of score 2 lesions in the Treponema-positive compared to the Treponema-negative group suggests that Treponema infection may be associated with more advanced stages of footrot and the possible presence of CODD infection. Similarly, Staton *et al*. reported that Treponema spp. were more prevalent in the later stages of footrot and CODD lesions[19]. However, in a Swedish study of slaughtered lambs with footrot, Treponema spp. was found in 89.7%, 94.7%, and 100% of the samples with scores of 0, 1, and 2, respectively, although the association between the footrot score and the presence of *Treponema* spp. was not significant.[38]

Our current findings highlight the dynamic interaction between Treponema infection and the severity of CODD lesions. The presence of Treponema appears to be associated with more advanced stages of the disease, emphasizing the need for targeted interventions in cases of Treponema positivity. Additionally, the higher prevalence of severe lesions in the Treponema-positive group underscores the importance of early detection and treatment to mitigate the impact of CODD on the affected sheep.

The sheep of the southern region exhibited a markedly higher prevalence of Treponema-positive cases, accounting for 88.1% (n = 37) in comparison with those of the northern regions sheep 7.1% (n = 3). This sharp contrast in infection rates between the north and south sheep of the region may be attributed to variations in environmental conditions, herd management practices, or potentially distinct strains of Treponema prevalent in each area.

Isolation of the Treponema spp. from deep tissues taken from footrot lesion of the sheep can be considered as a sign for the presence of clinical CODD in Iran, which may negatively affect sheep production, welfare, and the use of appropriate antibiotics. It has become apparent that the Treponemes must be considered as opportunistic infective agents and clinically manifest as new serious infectious diseases which are very difficult to treat. This detailed survey analysis in relation to Treponema status may provide a valuable insight for the development of targeted strategies for the management and control of CODD.

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Conflict of Interest

All authors disclose any financial and personal relationships with other people or organizations that might inappropriately influence or bias this work.

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