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Research Article

The Isolation of *Dichelobacter nodosus* and Identification by PCR from Ovine Footrot in Kars District, Turkey [1]

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Abstract

In this study it was aimed to isolation and identification by PCR of specific agent from ovine footrot in Kars district and thus determination of prevalence of disease. To this end, 8,970 sheep belong to 10 different flocks were examined clinically, and in 1532 of these (17.07%) were found lameness for various reasons. Out of 247 (2.75%) of these cases were evaluated to be footrot suspect clinically. Bacteria were isolated in 205 (82.99%) of the 247 samples that were cultured in an anaerobic environment due to the suspicion of footrot. When Gram stains and microscopic investigation was carried out on these isolates, 195 of them (95.12%) were found to be Gram negative rod-type bacteria. These isolates were subjected by polymerase chain reaction (PCR) using *Dichelobacter nodosus* specific primer and amplicons (440bp) of expected weight in 153 (78.46%) of isolates were found. Considering of these findings, it was concluded that prevalence of disease is high in sheep in Kars district.

Keywords: *Sheep, Footrot, Dichelobacter nodosus, Isolation, PCR*

Kars Yöresi Koyunlarında Piyeten Olgularından *Dichelobacter nodosus* İzolasyonu ve PCR İle İdentifikasyonu

Özet

Bu araştırmada, Kars yöresinde footrotlu koyunlardan hastalığın spesifik etkenin izolasyonu, PCR ile identifikasyonu ve böylece hastalığın prevalansının saptanması amaçlanmıştır. Bu amaçla 10 farklı sürüye ait 8970 koyun klinik olarak incelenmiş 1532'sinde (%17.07) çeşitli nedenlere bağlı topallık görülmüştür. Bunlardan 247'si (%2.75) klinik açıdan footrot şüpheli olarak belirlendi. Footrot şüphesiyle anaerobik ortamda kültürel olarak değerlendirilen 247 örneğin 205 inde (%82.99) bakteriyel izolasyon gerçekleştirilmiş, izolatların yapılan Gram boyama ve mikroskobik incelenmeleri sonucu bunların 195'i (%95.12) Gram negative çomak morfolojisinde bakteriler olarak görülmüştür. Bu izolatlar *Dichelobacter nodosus*'a spesifik primer kullanılarak polymerase chain reaction (PCR) a tabi tutulmuş ve 153'ünde (%78.46) beklenen ağırlıkta (440 bp) amplikonlar saptanmıştır. Bu bulgular dikkate alındığında, Kars yöresindeki koyunlarda hastalığın prevalansının yüksek olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Koyun, Piyeten, Dichelobacter nodosus, İzolasyon, PCR*

INTRODUCTION

Footrot is a specific contagious disease of sheep and goats, although it has been reported in cattle, horses, pigs, deer and mouflon. It is an infectious syndrome caused by synergistic action, where *Dichelobacter nodosus* is the main transmitting agent. Ovine footrot is characterized by the separation of keratinous hoof from the underlying tissue resulting in severe lameness, degraded body condition and reduced wool production [1,2]. *D. nodosus* is a rod shaped, Gram-negative, obligate anaerobic bacterium that has proteases and keratinases that are able to dissolve sheep hooves [3,4]. The primary predisposing factors for disease include environmental conditions as well as the

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host's genetics, immunity, diet and stocking rates [5,6].

Although footrot is widespread in many areas of the world where sheep are raised, it is particularly prevalent in temperate, rainy regions such as the UK, Australia and New Zealand [7]. Reports have been made in many countries regarding the disease's aetiology, pathogenesis, epidemiology, treatment, control and eradication. Turkey ranks seventh in the world with regard to sheep population. There are approximately 31 million sheep in Turkey, and 1.49% of them are raised in the region of Kars [8]. Approximately 70% of the people in Kars province work in the area of farming and animal husbandry. More than 90% of the sheep raised in the region are from the Morkaraman and Akkaraman breeds. Outside of the winter months, the sheep spend a bit more than half the year (from April to November) grazing in pastures. The animal owners and shepherds have very little knowledge or interest in foot diseases, so they do not conduct immunization, foot baths or hoof care. Furthermore, the government does not carry out any program to inform farmers about the disease or control and eradicate it. The goal of this study was to establish the status of footrot in sheep in the region of Kars, isolate the agent and identify using PCR. The study did not take into consideration the breed, age and diet of the sheep or environmental factors such as rainfall, moisture and type of terrain.

MATERIALS and METHODS

Reference Bacterial Strain

The reference strain of *D. nodosus* (ATCC 25549) was obtained from the Leibniz-Institut DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Study Region, Period and Sampling

This study was conducted on sheep raised in Kars province, which is in the Northeast Anatolia region of Turkey. The study was conducted on 8.970 sheep from 10 herds (herd size ranged from 800 to 1.100) grazing at various locations in the region from April 2013 to October 2014 (the pasture grazing period).

Clinical Investigation and Sampling Method

Of the 8.970 sheep that were evaluated from 10 different herds, 1.532 of them were found to have lameness for various reasons. These sheep were analysed with the scoring system recommended by Egerton and Roberts [9] for footrot lesions. Those sheep with a lesion score of 2 (interdigital dermatitis) to 4 (severe interdigital dermatitis and under-running of the hard horn of the hoof) were suspected to have footrot, and samples were collected from these 247 sheep using sterile cotton swab. These samples were transferred to a Stuart Transport Medium (Oxoid CM0111) [10], rapidly transported to the laboratory with an unbroken cold chain, and then immediately evaluated.

Isolation

All of the samples were cultured by streaking them in Eugon agar (BD Bacto, Sparks, MD, USA) and trypticase-arginine-serine agar (TAS) [11]. To assist in the growth of *Dichelobacter* spp. colonies, 5% defibrinated sheep's blood was added to the mediums. After the plates were streaked, they were incubated at 37°C for 4-5 days in a 2.5 liter jar (Merck) using an Anaerogen kit (Oxoid) to ensure anaerobiosis. Afterward, if the Gram stain and microscopic characteristics of the colonies that grew were similar to *D. nodosus* [12], passage was performed onto Eugon agar.

DNA Extraction of Bacterial Cultures

Standard methods were used to extract DNA from the bacteria colonies. Using a sterile toothpick, selected colonies of *D. nodosus* cells were prepared in 1.5 ml microcentrifuge tubes in 100 µl of sterile phosphate buffered saline (PBS). The tubes were placed in a boiling water bath for 10 min, cooled on ice for 5 min and centrifuged at 13.000 x g for 10 min. One microliter of the supernatant was used for PCR.

Detecting the fimA Gene of D. nodosus Using PCR

The primers *(Table 1)* and PCR settings used to identify the *fimA* gene of *Dichelobacter nodosus* were chosen according to Cagatay and Hickford [13]. A standard strain obtained from DSMZ was used for a positive control,

Table 1. *Primers used to amplify the fimA gene region of Dichelobacter nodosus*
Tablo 1. *Dichelobacter nodosus'un fimA gen bölgesinin amplifikasyonunda kullanılan primerler*

Primer	Sequence (5'→3')	Serogroup Specificity
Forward		
U1	ATCCCTGCATACAACGACTACAT	A, B, C, E, F, G, I and M (class I)
U2	GC TATTC CACAATAC CAAAACTACAT	D and H (classII)
Reverse		
D1	AC TCAAGAGAGAGGC TTTTAAGTAAG	B, C, G, E and M
D2	AGAGAGGCTTTCACATTTAAGAGC	A, F, I, E and M
D3	GTAC CGAAGTA CAC C TTTGATTG	D and H

while sterile distilled water was used for a negative control.

Analysis of the PCR Products

The PCR products were electrophoresed in 1% agarose gels, stained with nucleic acid stain (Safeview, NBS Biologicals Ltd) and visualized under UV illumination (UVP LMS-20E, Upland, CA, USA) and then photographed.

RESULTS

Clinical Examination

An evaluation was performed on 8.970 sheep from 10 flocks, and 1.532 of the sheep (17.07%) were found to have lameness for various reasons. The cause of lameness in the majority of the cases was hoof horn deformities, and clinical footrot was found to be the cause in 247 of these cases (16.12%). When compared to all of the sheep that were clinically evaluated in the study, the prevalence of footrot was found to be 2.75%.

Isolation Results

Bacteria were isolated in 205 (82.99%) of the 247 samples that were subjected to a bacterial culture in an anaerobic condition because footrot was suspected. When Gram stains and microscopic investigation was carried out on these isolates, 195 of them (95.12%) were found to be Gram negative rod-type bacteria. These isolates were subjected to PCR because *D. nodosus* was suspected.

PCR Detection of the fimA Gene

Of the 195 potential isolates that were subjected to a PCR, a 440-bp amplicon that was of the expected size for the *D. nodosus fimA* gene was identified in 153 of them (78.46%) *(Fig. 1)*.

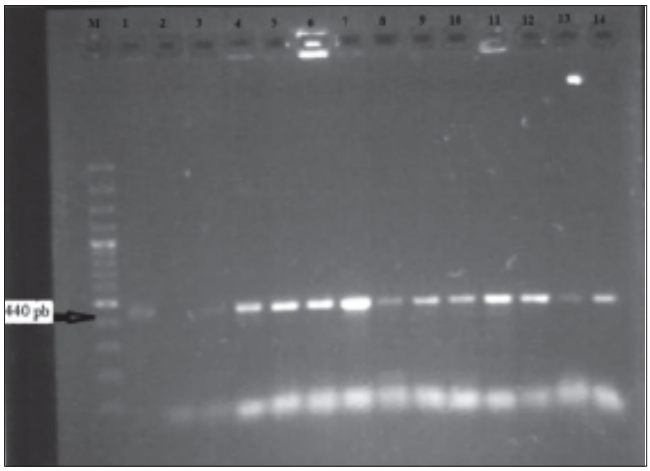

Fig 1. Specific PCR products of *Dichelobacter nodosus* from clinical samples of sheep with footrot. Lane M: 100 bp DNA marker, lane 1: positive control, lane 2: negative control, lane 3-14: *D. nodosus* positive samples

Şekil 1. Piyetenli koyunlara ait klinik örneklerden izole edilen *Dichelobacter nodosus* spesifik PCR ürünleri. M: 100 bp DNA marker, 1: pozitif kontrol, 2: negative kontrol, 3-14: *D. nodosus* pozitif örnekler

DISCUSSION

The disease of footrot in sheep is economically significant because it causes lameness, weight loss and wool production loss to various degrees in many countries around the world where sheep are raised. The prevalence of the disease has been reported at 12.54% [2] and 16.41% in Kashmir, India [14], 10% in the United Kingdom [15] and 3.1% in Bhutan [16].

Turkey is divided into seven different geographical regions based on climactic conditions and altitude. In spite of this variety of geography, there are very few studies that isolate and identify the agent for ovine footrot due to the difficulty of culturing many of the microbes that cause foot diseases in cloven hoof animals (anaerobiosis, fragility, mixed infection etc.) [17]. Previous studies have been largely focused on the healing effects of different diagnosis and treatment options using clinical radiological imaging. In a study that conducted a clinical and radiological investigation of 9.052 sheep in the Burdur region (an area in Turkey's Mediterranean region) during the sheep pen and pasture seasons in order to analyse the distribution and environmental factors of foot diseases in sheep, Avki et al.[18] found that 1.576 animals (16.30%) had foot disease, that 13.46% of these diseases were hoof deformities, and that 2.55% of them were ovine footrot. In another study that conducted a clinical and radiological evaluation of foot diseases observed in sheep raised in the regions of Kars and Iğdır [19]; 4,230 sheep were examined in the pasture season and 3.770 were examined in the pen/stall period for a total of 3.770 sheep. Foot disease was found in 1.080 (25.51%) and 520 (13.76%) sheep, respectively. Hoof horn deformities were the most prevalent in both seasons, while the prevalence of footrot was found to be 2.83% in the pasturing period and 0.82% in the pen/stall period.

This study evaluated 8.970 sheep from 10 flocks being raised in various parts of Kars province, and 1.532 of them (17.07%) were found to have lameness for various reasons. The cause of lameness in the majority of the cases was hoof horn deformities, while clinical footrot was found to be the cause of lameness in 247 of these cases (16.12%). When compared to all of the sheep that were clinically evaluated in the study, the prevalence of footrot was found to be 2.75%. This percentage was lower than some of the aforementioned studies [2,14,15] but quite close to the results of other studies [16,19]. It is known that some factors related to the host animal (such as breed and immunity) as well as certain environmental factors (such as rain, temperature and moisture) have an effect on the natural progression of the disease. For example, the average annual rainfall in the UK and India is much higher than that of Kars province, while Bhutan's average annual rainfall is quite similar to

this area. The prevalence figures obtained in this study prove that atmospheric conditions such as rainfall, temperature and moisture have a definite effect on the progression of the disease.

Isolating *D. nodosus* is an extremely difficult and time consuming process, partly because of the fastidious nature of this strict anaerobe, but also because of the large number of different bacteria in the microflora of the footrot lesion [20,21]. Furthermore, taking samples correctly and quickly transporting them to the laboratory under suitable conditions is absolutely crucial to obtain an accurate isolation rate. Bacteria were isolated in 205 (82.99%) of the 247 samples that were cultured under anaerobic conditions due to suspicion of footrot. This isolation rate can be affected by how the samples are taken and transported to the laboratory, the streaking stages and the incubation conditions. When Gram stains and microscopic investigation were carried out on the isolates, 195 of them (95.12%) were found to be Gram negative rod-type bacteria. These isolates were subjected to PCR because *D. nodosus* was suspected. Of the 195 isolates subjected to PCR, 153 (78.46%) of them were found to be *D. nodosus*. It is known that cases with ovine footrot feature bacterial complexity [6] and that *Fusobacterium necrophorum* has a synergistic effect in the formation of the lesions [3,22]. This leads to the conclusion that the isolates that could not be identified as *D. nodosus* may be other agents that are part of the aetiology of the disease.

This was the first study to be conducted to establish the status of footrot in sheep from the region of Kars, isolation the agent and identify it with PCR. The results have shown that the disease is significantly prevalent in the sheep of the region and *D. nodosus* was isolated and identified in a high percentage of the subjects. The goal of subsequent studies should be to identify the serogroup and serotypes of the strains of *D. nodosus* that are isolated. This is necessary in order to develop a vaccination against the disease. In view of the disease's infectiousness and economic impact, it is critically important that a range of programs and activities be carried out, including vaccination, hoof care, foot baths, evaluating the effects of environmental factors on the disease, separating sick animals from healthy ones, treating sick animals as soon as possible, and training sheep ranchers about these topics.

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