Isolation and identification of *Dichelobacter nodosus* and *Fusobacterium necrophorum* using the polymerase chain reaction method in sheep with footrot

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Abstract

Footrot is an important infectious disease of small ruminants leading to severe economical losses. The aim of the present study was to determine isolation and identification rates of *Dichelobacter nodosus* and *Fusobacterium necrophorum* in the culture techniques and reveal the specificity and sensitivity of the culture technique based on the polymerase chain reaction (PCR) method in sheep with footrot. Dry swabs and swabs with Amies medium from 83 sheep were subjected to PCR and culture analyses. In dry swabs, 4 samples were positive for *F. necrophorum* and all were negative for *D. nodosus*. Colonies in Eugon and Fusobacterium selective agars from swabs with Amies medium were evaluated. Polymerase chain reaction analysis was conducted on macroscopically and microscopically unidentified samples. The positivity rate was 55.4% for *D. nodosus* and 69.8% for *F. necrophorum* in cultures from Fusobacterium selective agars. The positivity rate for *D. nodosus* in Fusobacterium selective agars was higher than that in Eugon agar. Performing PCR and culture methods increased positivity as compared to performing them alone. In comparison with the PCR method, culturing in Fusobacterium selective agars had moderate sensitivity and low specificity for *D. nodosus* (71.7 and 28.7%) and *F. necrophorum* (61.3 and 80.0%), respectively. In conclusion, Fusobacterium selective agar (without antibiotics) for isolation and identification of *D. nodosus* is superior to Eugon agar. *Fusobacterium necrophorum* should also be considered as a provoking agent for footrot in small ruminants. The PCR method on culture increases elucidation of definitive aetiology.

Anaerobe agents, PCR, selective medium, sensitivity, specificity

Footrot is a contagious disease of sheep and goats, affecting the capsula ungulae and the interdigital region (Sagliyan et al. 2003; Sertkaya and Sindak 2004; Cagatay and Hickford 2006; Raadsma and Egerton 2013). Its mortality rate is reported to be 40%, whereas its morbidity rate is highly variable (8–100%) (Sagliyan 2003). The outbreak occurs mostly in wet seasons (Allworth 2004; Sertkaya and Sindak 2004) because the disease spreads via contacting mud and faeces as well as consuming roughages contaminated with purulent discharges in grasslands (Raadsma and Egerton 2013).

Dichelobacter nodosus and *Fusobacterium necrophorum* are the primary and secondary agents, respectively, when both are involved (Sagliyan et al. 2003; Sertkaya and Sindak 2004; Raadsma and Egerton 2013). *Dichelobacter nodosus* is a Gram negative, non-spore-forming rod, and obligate anaerobic bacterium (Garrity et al. 2005; Raadsma and Egerton 2013). Hoof agar, TAS (Trypticase arginine serine) agar and Eugon agar are suggested for its isolation (Stewart and Claxton 1982). Fimbria, protease, and elastase structures are known determinants of its virulence. The known *D. nodosus* virulence factors are its type IV fimbria and extracellular serine proteases (Garrity et al. 2005). *Fusobacterium necrophorum* is a Gram negative, non-spore-forming rod, and obligate anaerobic bacterium (Timoney et al. 1988).

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Inflammation begins in the interdigital region and skin where capsule ungulae contacts at onset. Signs include hair loss in the region, sensitivity and reddishness of skin, and presence of serous exudate and necrosis in the neighbouring tissues. In severe cases, inflammation diffuses from the interdigital region to abaxial wall of capsulae ungulae. The hoof is affected by lesion and purulent exudate (Sagliyan et al. 2003). The degree of severity of the disease is based on the lesion intensity, which is as follows (Stewart and Claxton 1982):

Score 1; moderate inflammation causing erosion of interdigital skin epithelium,

Score 2; necrotic inflammation covering the interdigital region and partially or completely the axial wall of the hoof,

Score 3; necrotic inflammation extending to the abaxial wall of the hoof,

Score 4; hoof structure is disturbed due to lesion expanded to the abaxial wall,

Score 5; laminae and other soft tissues are necrotized.

Production loss, preventive measures, and treatment are very expensive (Raadsma and Egerton 2013). Wool/mohair, milk, body condition, body weight, and lambing losses are very critical (Sagliyan 2003; Sagliyan et al. 2003). Reports from the UK and Australia show that footrot cost 24 million pounds and 18.4 million dollars between 2005 and 2006, respectively (Dhungyel et al. 2013). The present experiment was conducted to determine the isolation and identification rates of *D. nodosus* and *F. necrophorum* in the culture techniques and to reveal the specificity and sensitivity of the culture technique based on the polymerase chain reaction (PCR) method in sheep with footrot.

Materials and Methods

Sample collection

The study was conducted on 83 sheep suspected with footrot based on clinical signs. Samples were collected using dry swabs and swabs with Amies medium. The ATCC 25549 *D. nodosus* and ATCC 25285 *F. necrophorum* were used as positive controls.

After consulting the State Agriculture Department between April and November 2013 about footrot occurrence as herd basis by provinces, samples were taken from sheep and goats exhibiting footrot greater than Score 2 using swabs with Amies medium for culture and sterile dry swabs for PCR analysis. Swabs were collected and stored at 4 °C for culture and at -20 °C for PCR analysis.

Bacteriology

Samples were cultured on Eugon agar enriched with 5% defibrinated sheep blood for isolation of *D. nodosus* and Fusobacterium selective agar (FSA) with 5% defibrinated sheep blood and other components (*i.e.,* hemin, tween 80, and dithioeritrol), but without antibiotic supplements (*i.e.,* neomycin, josamycin, and vancomycin) for isolation of *D. nodosus* and *F. necrophorum*. Prior to culture these media were kept in jars along with a commercial kit (AnaeroGen™, Oxoid Ltd, Basingstoke, Hampshire, England) to provide anaerobiosis. Colonies were evaluated under a microscope (BX43, Olympus, Tokyo, Japan) after incubation at 37 °C for 3 d in terms of morphology, haemolytic properties, incubation needs, and Gram staining (Quinn et al. 2004). Colonies were then transferred to sterile 1.5 ml tubes with sterile phosphate buffered saline (PBS) for PCR analysis.

DNA extraction of swab samples

The DNA was extracted from dry swabs using the DNA extraction kit (DNeasy Blood and Tissue kit, Qiagen, Hilden, Germany). Briefly, dry swabs were placed in sterile 1.5-ml tubes with sterile PBS and vortexed. A 100-µl sample was used for DNA extraction according to the manufacturer's recommendations.

DNA extraction of bacterial cultures

Bacterial DNA was extracted from colonies using the DNA extraction kit. Colonies were collected from agar and suspended in sterile 1.5-ml tubes with sterile PBS. Colonies were thawed and vortexed. A 100-µl sample was used for DNA extraction according to the manufacturer's recommendations. Primers specific to *fimA* gene region of *D. nodosus* and *lktA* gene region of *F. necrophorum* (Tables 1) were used in the PCR analysis (Cagatay and Hickford 2006; Bennett et al. 2009a).

Polymerase chain reaction

The mixture (20 µl) for *D. nodosus* was prepared to contain each primer (0.25 mM), each dNTP (200 mM), DNA (1 µl), Taq DNA polymerase (1 U), and PCR buffer. After a 2-min thermal cycle (TechneTC-5000, Thermo

Scientific, UK) at 94 °C for 40 cycles, aliquots were processed at 94 °C for 30 s for denaturation, at 62 °C for 30 s for binding, at 72 °C for 50 s for elongation, and at 72 °C for 5 min for final elongation. Specimens were then placed in gel (1%) and images were obtained in UV transilluminator (GL-500, Geneline, Giangarlo Scientific Co, Inc., Pittsburgh, PA). All samples of PCR amplification products (10 µl) were subjected to electrophoresis. The DNA was visualized by UV fluorescence after staining with ethidium bromide. The PCR amplification products with 450 bp were considered *D. nodosus* positive (Fig. 1) (Cagatay and Hickford 2006).

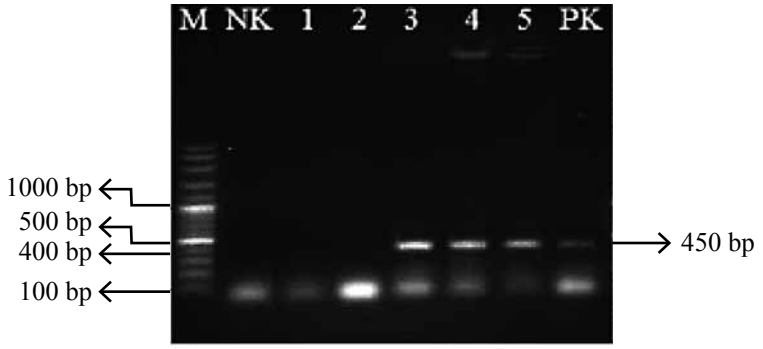

Fig. 1. Determination of *D. nodosus* in PCR. M: Marker (DNA Ladder Plus, Fermantas, Lithunia), NK: Negative control, 1-5: *D. nodosus* positive samples, PK: Positive control.

Similarly, the mixture (20 µl) for *F. necrophorum* was prepared to contain each primer (0.25 µM), each dNTP (150 µM), extracted DNA (1 µl), *Taq* DNA polymerase (0.5 U), and PCR buffer. After a 5-min thermal cycle at 94 °C for 35 cycles, aliquots were processed at 94 °C for 30 s for denaturation, at 59 °C for 30 s for binding, at 72 °C for 30 s for elongation, and at 72 °C for 5 min for final elongation. Specimens were then placed in gel (1%) and images were obtained in UV transilluminator. All samples of PCR amplification products (10 µl) were subjected to electrophoresis. DNA was visualized by UV fluorescence after staining with ethidium bromide. PCR amplification products with 400 bp were considered *F. necrophorum* positive (Fig. 2) (Bennett et al. 2009a).

Fig. 2. Determination of *F. necrophorum* in PCR. M: Marker (DNA Ladder Plus, Fermantas, Lithunia), NK: Negative control, 1-9: *F. necrophorum* positive samples, PK: Positive control.

Data analysis

The frequency procedure was employed in data analysis to attain the isolation and identification rates by the culture technique. Moreover, receiver operating characteristic (ROC) curves were developed to determine the sensitivity (the ability to detect an ill animal), specificity (the ability to avoid detecting a healthy animal), positive likelihood ratio (low specificity or the odds of an ill animal when the test is positive), and negative likelihood

ratio (low sensitivity or the odds of an ill animal when the test is negative) of culture diagnosis on PCR analysis (MedCalc Statistical Software version 13.1.2, Ostend, Belgium).

Results

All samples collected by sterile dry swabs were negative for *D. nodosus*, whereas 4 samples (4.8%) were positive for *F. necrophorum* in PCR analysis.

All suspected samples cultured in Eugon agar were negative for *D. nodosus* in the PCR analysis. Among the suspected samples cultured in FSA, *D. nodosus* was positive in 46 samples (55.4%) and *F. necrophorum* was positive in 58 samples (69.8%) in the PCR analysis (Table 1). Both *D. nodosus* and *F. necrophorum* were negative and positive in 18 and 39 cultured samples, respectively. In 19 cultured samples, *D. nodosus* was negative, whereas *F. necrophorum* was positive. In 7 cultured samples, *D. nodosus* was positive,

Table 1. Primers specific to *fimA* gene region of *D. nodosus* and *lktA* gene region of *F. necrophorum.*

Primer	Sequence
D. nodosus (*fimA* gene)	
Forward	
U1	5' - ATCCCTGCATACAACGACTAC AT - 3'
U2	5' - GCT ATT CCACAATACCAAAACTAC AT - 3'
Reverse	
D1	5' - ACT CAA GAG AGA GGCTTTTAAGTA AG - 3'
D2	5' - AGA GAG GCTTTCACATTTAAGAGC - 3'
D3	5' - GTACCGAAGTACACCTTT GAT TG - 3'
F. necrophorum (*lktA* gene)	
Forward	5' –ACAATCGGAGTAGTAGGTTC - 3
Reverse	5' - ATT TGGTAACTGCCACTG C - 3'

Table 2. PCR analysis of suspected samples cultured in FSA by the province.

Province	*D. nodosus* +	*F. necrophorum* +	*D. nodosus* *F. necrophorum* +	*D. nodosus* *F. necrophorum* -	*D. nodosus*+ *F. necrophorum* -	*D. nodosus* - *F. necrophorum* +
A	1	-	3	1	1	-
B	-	1	-	3	-	1
C	-	1	-	3	-	1
D	2	1	3	-	2	1
E	-	1	-	3	-	1
F	2	-	-	3	2	-
G	-	-	-	5	-	-
H	1	4	5	-	1	4
I	-	3	-	-	-	3
J	-	5	7	-	-	5
K	-	1	11	-	-	1
L	1	2	10	-	1	2
	46 (55.4%)	58 (69.8%)	39 (46.9 %)	18 (21.6 %)	7 (8.4%)	19 (22.8%)

FSA - Fusobacterium selective agar

whereas *F. necrophorum* was negative. Positivity for either bacterium in the PCR analysis was considered footrot (Table 2).

The sensitivity and specificity of FSA agar as compared to the PCR method were 71.7 and 28.7% for *D. nodosus* and 61.3 and 80.0% for *F. necrophorum*, respectively (Plate I, Fig. 3).

Discussion

While *D. nodosus* is the primary pathogen causing footrot, *F. necrophorum* invades the interdigital areas secondarily and eases a continuing invasion of *D. nodosus*. Therefore, the infection should occur through a synergistic action with *F. necrophorum*. Although culture is a common method for pathogen identification, bacteria causing footrot are obligate anaerobes, sensitive to dryness, requiring special medium, which makes the isolation and identification difficult, especially when the sampling site is contaminated with other pathogens (Rood et al. 1996; Bennett et al. 2009a; Tadepalli et al. 2009). Thus, enrichment of the medium to provide selectivity is critical for colony formation. Eugon agar enriched with 5% sheep blood for isolation of *D. nodosus* and FSA for isolation of *F. necrophorum* are commonly used. However, Eugon agar is shown to be inefficient for isolating *D. nodosus*. Thus, addition of hemin, Tween-80, Na_2HPO_4, KH_2PO_4, $MgSO_4$, and dithiothretiol to Brucella agar enhances colonization of anaerobic bacteria, which adversely affects colonization of aerobic bacteria (Nitzan et al. 1994; Noel et al. 2005). Tween-80 stimulates enzyme secretion based on the degree of bacterial cell wall permeability increase (Lee et al. 2003). Thus, we used FSA medium with hemin, Tween 80, Na_2HPO_4, KH_2PO_4, $MgSO_4$, and dithiothretiol and without antibiotics (vancomycin, josamycin, neomycine) to isolate *D. nodosus*. Indeed, more *D. nodosus* was isolated from colonies in FSA than in Eugon agar, which could be attributed to the enrichment of agar with hemin and Tween 80 (Nitzan et al. 1994; Lee et al. 2003; Noel et al. 2005). For being specific to *F. necrophorum*, its isolation in FSA was easier than of *D. nodosus* (Morgenstein et al. 1981). However, there is lack of comparison with Eugon agar because only FSA was used for isolation of *F. necrophorum*.

Rood et al. (1996) conducted a typing on 771 frozen *D. nodosus* isolates in 4% hoof and Eugon agars, followed by biochemical tests and reported difficulty in isolation and slow growth rate of *D. nodosus* in conventional methods. The PCR is a commonly used method for isolation and identification of a number of bacteria after culture, including *D. nodosus* and *F. necrophorum* (Ghimire et al. 1998; Moore et al. 2005; Zhou et al. 2010; Petrov and Dicks 2013). In a similar experiment, Cagatay and Hickford (2006) incubated swab samples in hoof agar and Eugon agar and performed PCR on suspected samples for *D. nodosus fimA* region. In the present study, colonies grown on Eugon agar were negative for *D. nodosus* and those grown on FSA were positive for *D. nodosus* and *F. necrophorum*. These observations and PCR confirmations are in agreement with previous studies (Ghimire et al. 1998; Moore et al. 2005; Petrov and Dicks 2013). The positivity frequency is reported to be greater in the PCR analysis performed on cultured colonies than in the PCR analysis performed directly on dry swab samples (Ghimire et al. 1998; Moore et al. 2005; Cagatay and Hickford 2006; Petrov and Dicks 2013).

The PCR method is considered golden standard for bacterium identification. In the present study, despite being grown on FSA agar, sensitivity of culturing on FSA agar was moderate for *F. necrophorum* and *D. nodosus*. However, specificity of FSA agar was expectedly low. Identification of *F. necrophorum* and *D. nodosus* differs in the literature. Bennet et al. (2009b) examined 148 hoof samples and reported that positivity for *F. necrophorum* and *D. nodosus* was 53% (n = 79) and 5% (n = 7) in the PCR analysis employing *lktA* gene and *fimA* gene, respectively. *Fusobacterium necrophorum* and *D. nodosus* are considered

primary and secondary causative agents for septic laminitis in cattle and sheep footrot. Petrov and Dicks (2013) conducted an experiment to verify the etiological role of *F. necrophorum* and *D. nodosus* in hoof thrush in horses. Out of 28 swab samples collected from 8 healthy horses and 8 horses with hoof thrush, there were colony formations in 6 samples (5 from affected horses and 1 from ahealthy horse). Biochemical tests revealed *F. necrophorum* subsp. *necrophorum, F. necrophorum* subsp. *funduliforme*, and *F. equinum* in colonies. However, PCR analyses for *lktA* and *fimA* gene regions showed positivity for *Fusobacterium* spp. in all colonies, but not for *D. nodosus*. They concluded that *F. necrophorum*, but not *D. nodosus,* was a causative agent for hoof thrush in horses.

In the experiment of Zhou et al. (2009), the positivity rate for *F. necrophorum* was 13/14, 6/6, and 9/9 swab samples collected from 14 sheep, 6 goats, and 9 cattle with septic laminitis using primers specific to *lktA* gene region in the PCR analysis. These suggest that *F. necrophorum* should also be considered a causative agent for footrot in small ruminants. Bennet et al. (2009c) examined samples from healthy goats and 24 goats with footrot by the PCR method. Healthy goats were negative for *F. necrophorum* and *D. nodosus*, whereas affected goats had positivity for *D. nodosus* (62.5%; 15/24) and *F. necrophorum* (33.3%, 8/24). Of the samples positive for *F. necrophorum*, 7 were also positive for *D. nodosus* (87.5%). In another experiment by Bennet et al. (2009a), positivity for *F. necrophorum* was reported in one sample in 50 healthy sheep. All healthy sheep were negative for *D. nodosus*. In 17 samples from sheep with footrot (n = 42), there was positivity for both *F. necrophorum* and *D. nodosus*. The positivity of one healthy animal was attributed to sampling from a contaminated region. Studies conducted in Kashmir, positivity for *D. nodosus* using the PCR analysis on swab samples was reported to be 54% (n = 370) (Rather et al. 2011) and 61% (n = 200) (Bhat et al. 2012) in sheep with footrot.

In another study conducted in Germany with swab samples incubated on Eugon agar (n = 13), the culture was positive in 11 samples, of which 6 were positive for *D. nodosus* in the PCR analysis (Zhou et al. 2010). A more comprehensive experiment was conducted by Moore et al. (2005) on 39 farms in England and Wales in 2000–2004. The researchers selected two healthy and six affected sheep from each farm, totalling samples from 77 healthy and 193 affected sheep. The positivity rate for *D. nodosus* was 11/77 in healthy sheep and 105/193 sheep with footrot. Of the collected samples, 263 were subjected to PCR analysis and culture, which showed positivity in 112 samples and negativity in 110 samples. In 39 samples, the PCR analysis was positive and the culture was negative. In 2 samples the PCR analysis was negative and the culture was positive. These figures suggest that PCR has 1% false-negative and 15% true-positive rates compared to culture, indicating superiority of the PCR method to the culture in agent identification. Our findings for *D. nodosus* (Moore et al. 2005; Bennett et al. 2009a; Rather et al. 2011) and *F. necrophorum* (Bennett et al. 2009a, 2009c; Zhou et al. 2009) were inconsistent with literature. Inconsistency may be caused by the severity of disease, involvement of other bacteria, sampling collection protocol, and the production system. Because the bacterium is an obligate anaerobe sensitive to environmental conditions and potential contamination of the sampling site, performing both culture and PCR analysis makes identification of *D. nodosus* and *F. necrophorum* more accurate than performing each of these methods alone. This approach helps determine the effective prevention and treatment programme (Zhou et al. 2009).

In summary, our data suggest that *F. necrophorum* should also be considered as a provoking agent for footrot in small ruminants in addition to *D. nodosus*. The use of enriched agar increases the isolation success of *D. nodosus*. The FSA agar has low sensitivity and specificity for detecting *D. nodosus* and *F. necrophorum*. Employment of the PCR method on culture could increase definitive aetiology, and consequently determination of effective protocols of prevention and treatment.

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