Assessment of two different methods for sampling and detection of *Dichelobacter nodosus* and *Fusobacterium necrophorum* in dairy cows in Eastern Slovakia

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**ABSTRACT**
The aim of our study was to find the most appropriate way of sample collection from cattle feet as well as to assess simple and effective sample processing, including DNA extraction for reliable diagnosis of bacteria *Dichelobacter nodosus* and *Fusobacterium necrophorum*. 11 clinically healthy cows were included in the study, from which swabbing samples (2 types: surface swab and deep swab) were taken. Two isolation methods were used for DNA extraction: 1. freezing and boiling the samples, 2. commercial kit (Roche). PCR analysis of the samples has not shown any variations in the detection ratio of *D. nodosus* and *F. necrophorum* at different swabbing methods. The highest sensitivity of the detection of both bacteria was reached with a cultivation of samples in AB with subsequent extraction of DNA with freezing and boiling. The cultivation in anaerobic broth resulted in the detection rate of *D. nodosus* and *F. necrophorum* in over 95% and 27%, respectively. To conclude, the simple ‘surface’ swab is sufficient to detect studied pathogens, the most appropriate method of DNA extraction has proven to be freezing and boiling of the sample.

**1. Introduction**

Foot rot (interdigital phlegmon) is a significant health disorder of dairy cattle caused by bacteria *Dichelobacter nodosus* and *Fusobacterium necrophorum* (Roberts and Egerton 1969; Langworth 1977; Weaver et al. 2005; Wani and Samanta 2006; Bennet et al. 2009b). In many cases, it could cause a critical condition for breeders due to fast onset of severe clinical signs, such as severe pain, lameness and in the end a long-term recumbency of the animal (Reinöhl-DeSouza and Kofler 2006). Effected animals have a reduced feed intake which has a negative influence/impact on the farm economics, thus, it is necessary to detect and diagnose these animals immediately (Hernandez et al. 2002). Confirmation of foot rot in the farm should result in rapid application of therapy/prevention measures both to heal affected animals and to protect other animals from infection (Morck et al. 1998; Weaver et al. 2005; Reinöhl-DeSouza and Kofler 2006; Apley 2015).

*F. necrophorum* is an obligatory anaerobic gram negative bacterium, pleomorphic or bacillary shaped. It is part of the natural microflora of the gastrointestinal tract of animals and humans (Langworth 1977; Egerton 2002; Narayanan et al. 2002a, Nagaraja et al. 2005; Bennett et al. 2009b, 2009b). Exotoxin – leukotoxin (*lktA*), produced by *F. necrophorum* subspecies *necrophorum*, is a major virulence factor within biotype A and it is a primary toxin to ruminant leucocytes. It can induce apoptosis of immune cells and at higher concentrations damage leucocytes, moreover it is more active against polymorph nuclear leucocytes than to lymphocytes (Narayanan et al. 2002a, 2002b; Nagaraja et al. 2005).

The epidermal tissue of cattle feet is a natural environment for *D. nodosus*, where it can persist for up to two years but in the environment, it survives for less than seven days (Altenbrunner-Martínek 2011). *D. nodosus* is a bacillary shaped gram negative, obligatory anaerobic bacteria. *D. nodosus* was divided based on differences between fimbrial proteins and genes into 10 serogroups (A – I, M) and these were subdivided into 19 serotypes (Bhat et al. 2012). The mechanism explaining synergistic relationships of *F. necrophorum* and *D. nodosus* can run through the production of *lktA* by *F. necrophorum* (Narayanan et al. 2001), which is involved in the creation of an immunocompromised environment of the skin which is optimal for growth and activity of *D. nodosus*. Leukotoxin – mediated effect is the likely cause of foot rot since *D. nodosus* is known to be susceptible to attack the immune system (Roberts and Egerton 1969). In return, the virulent strains of *D. nodosus* are able to produce a protease enzyme, causing hydrolysis of keratin that results in disruption of the skin structure (Thomas 1962). The disrupted skin is a medium enriched with nutrients and growth factors suitable for further propagation of *F. necrophorum* leading to a necrotic tissue breakdown (Bennett et al. 2009a, 2009b).

In the last decades, a fast, accurate method of reverse dot blot hybridization and PCR was discovered and introduced for detection of anaerobic bacteria (La Fontaine et al. 1993; Zhou et al. 2001). Meanwhile, the development of PCR ensured a classification of various strains of *D. nodosus* into serogroups (with amplification of the variable gene region for fimbrial subunit *flmA*) (Dhungyel et al. 2002; Wani and Samanta 2006).
Gilhuus et al. 2013). Primers detecting lktA gene, which determines the pathogenicity, are used for diagnostics of virulent strains *F. necrophorum* (Zhou et al. 2009; Bennet et al. 2009b; Bennet et al. 2010). A necessary step in accurate diagnostics is a correct and optimal collection and processing of samples.

The aim of our study was to find the most appropriate way of sample collection from the cattle foot as well as to assess simple and effective sample processing, including DNA extraction for reliable diagnosis of bacteria *D. nodosus* and *F. necrophorum*.

2. Material and methods

2.1. Sample collection

Samples were collected on a dairy farm located in Eastern Slovakia. Dairy cows (*n* = 11) were clinically healthy and free of symptoms of lameness, examination in the claw crush showed no signs of claw diseases. For sampling swabs, Amies Agar Gel Medium Transport Swabs (Sarstedt) were used. To test the optimal method of sampling, swabs were obtained from dairy cows in two ways:

1. ‘Surface‘ swab (SS) of planter interdigital space of hoof, not preceded by cleaning or other action performed on the feet before sampling. This method is very quick and easily enforced even during milking without the need of limb fixation.
2. In the ‘deep’ swab (DS), the interdigital space of the same leg used for a ‘surface swab’ was firstly thoroughly washed with water and then dried up with a paper towel. The swab of the interdigital skin followed using strong pressure on the skin.

2.2. Sample processing and DNA extraction

From each animal, four swabs were taken (2 × SS and 2 × DS). One of each pair of samples were incubated in 2 ml of PBS for 48 h at 4°C. At the end of the incubation period, 2 ml of PBS were divided into 1 ml to 1.5 ml tubes. The second swabs were incubated in 1.5 ml anaerobic broth (AB – Anaerobic Basal broth, HiMedia) for 48 h at 37°C under anaerobic conditions created by the Anaerocult (Merck). Immediately after the incubation, the extraction of DNA was processed.

Two isolation methods for DNA extraction were used. The first method was a simple, quick and inexpensive method, where the sample was frozen and then quickly boiled. The second method used for extraction of DNA was a commercial kit (High Pure PCR Template Preparation Kit – ROCHE). 1 ml of PBS and 1.5 ml of AB, in which the samples were incubated or cultivated from the surface (SS) and deep (DS) swabs, were used for DNA extraction by freezing and boiling. Briefly, after a centrifugation (Eppendorf Centrifuge 5418) of the samples (5000 x g for 5 min at room temperature), the resulting sediment was resuspended in 500 µl of saline and re-centrifuged (5000 x g for 5 min at room temperature). After removal of the supernatant, the sediment (pellet) was resuspended in 100 µl distilled water. Subsequently, the sample was frozen at −70°C for 10 min and boiled for 5 min at 100°C. Finally, the samples were centrifuged at 13,900 x g for 5 min at room temperature. The supernatant containing the DNA was then transferred to a clean tube. From the second ml of PBS, the DNA was isolated with using a commercial kit. The concentration of DNA in the samples were measured with NanoDrop™ 8000 Spectrophotometer, Thermo Scientific. The purity of isolated nucleic acids was determined by the ratio of the measured absorbance A260/280. The sample of DNA without contamination of proteins and other organic substances was considered to be pure when the ratio of the measured absorbance was between 1.8 and 2.0. Values below 1.8 indicate sample contamination. Subsequently, the sample with the isolated DNA was stored at −20°C until the testing.

2.3. PCR and electrophoresis

For diagnostics of *D. nodosus* specific primers for detection of gene coding 16S rRNA, which is by its sequence specific to the bacterial species, were designed. Diagnostics of *F. necrophorum* were done with primers specifically designed for gene region *leucotoxin A* (*lktA*). Based on the known gene sequences and the applications of general principles of selecting the specific primers, the primers were designed with the use of a software Primer3 (SAS EMBnet node, EMBnet Slovakia). Primers were synthesized and delivered by Generi Biotech (hradecky Kralove, Czech Republic, Tables 1 and 2). Conditions of the amplification and the composition of the reactive solution are presented in Tables 3 and 4.

DNA used for optimization of the PCR and also as a positive control was isolated from anaerobic cultures of lyophilized bacterial strains *D. nodosus* (DSM 20708) from collections in Germany (Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and *F. necrophorum* (CCM 5981) from the collection in Brno (CCM Czech Collection of Microorganisms, Brno, Czech Republic). Extraction of DNA was done with isolation kit ROCHE. As a negative control for PCR qH2O was used.

PCR was evaluated by agarose electrophoresis. Separation of DNA was performed in 2% agarose gel and the DNA was

| Table 1. Primer sequence for *Dichelobacter nodosus* (16S rRNA). |
|---------------------|---------------------|
| Primer              | Name                | Sequence (5’–3’) |
| Forward             | D.N.16S_rRNA_A      | GCTAAGGAAAAAGCACCCGGC |
| Reverse             | D.N.16S_rRNA_R      | GTTTCTACCACCGCTTTGC |

| Table 2. Primer sequence for *Fusobacterium necrophorum* (*lktA*). |
|---------------------|---------------------|
| Primer              | Name                | Sequence (5’–3’) |
| Forward             | F.N.lktA_A          | TTGGGAGCCGAGTGCCAG |
| Reverse             | F.N.lktA_R          | CTCCGGCTGCAAGAATTCCA |

| Table 3. Composition of reaction mixture. |
|------------------------------------------|
| Components                                | Amount (µl) |
| Dream Taq PCR Master Mix (Fermentas) (2X) (Thermo Fisher Scientific, Microbiology Division Czech Republic, Brno, Czech Republic) | 10 |
| qH2O (Thermo Fisher Scientific, Microbiology Division Czech Republic, Brno, Czech Republic) | 7 |
| Forward primer (10 µM) | 1 |
| Reverse primer (10 µM) + sample DNA | 10 |
| | 20 |
The results were statistically evaluated with GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego.

2.4. Statistical evaluation

The results were statistically evaluated with 'GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego.

Table 4. Conditions of amplification.

| Process            | Temperature (°C) | Time  | Cycle count |
|--------------------|------------------|-------|-------------|
| Denaturation 1     | 95               | 3 min | 1 – start   |
| Denaturation 2     | 95               | 30 s  | 35          |
| Annealing          | 57               | 30 s  |             |
| Elongation         | 72               | 30 s  |             |
| Final elongation   | 72               | 10 min| 1 – end     |

Figure 1. The PCR product of 16S rRNA of D. nodosus generated from field samples. L – Ladder 50 bp, K+ – positive control, K– – negative control with qH2O. Column 1–3 samples for D. nodosus, column 1 – the method of freezing and boiling from PBS is positive, column 2 – the method of freezing and boiling from AB is positive, column 3 – the method with high pure PCR template preparation kit is positive.

visualized by the GelRed™ (Biotium, Inc.) fluoroscein dye. The amplicon resulting from the PCR of the 16S rRNA transcription of the D. nodosus was detected in 295 bp and the lktA for F. necrophorum was detected in 362 bp. As molecular weight standards a 50 bp GeneRuler DNA Ladder was used (Thermo Fisher Scientific) (Figures 1 and 2).

Table 5. Detection rate (%) of D. nodosus and F. necrophorum in surface/deep swabs by using three different diagnostic methods.

|          | Surface | Deep | FET | Surface | Deep | FET |
|----------|---------|------|-----|---------|------|-----|
| PBS      | 50.3    | 72.5 | NS  | 5.12    | 2.52 | NS  |
| AB       | 95.2    | 97.5 | NS  | 27.5    | 27.5 | NS  |
| ROCHE    | 90.1    | 87.5 | NS  | 15.4    | 17.5 | NS  |

Note: FET: Fisher's exact test; NS: non significant.

Table 6. Detection rate (%) of D. nodosus and F. necrophorum in all swabs by using three different methods for DNA processing.

|          | PBS    | AB     | ROCHE   | FET   |
|----------|--------|--------|---------|-------|
| D. nodosus | 61.3a  | 95.1b  | 88.8b   | P<.05 |
| F. necrophorum | 3.75a  | 27.5b  | 16.3b   | P<.05 |

Note: FET: Fisher’s exact test; a, b: means with different letters differ (P<.05).

California USA’, using Fisher’s exact test. The differences were considered significant when P<.05.

3. Results

None of the diagnostic procedure for PCR detection of D. nodosus and F. necrophorum showed a significant difference in strain detection between two different swabbing methods (Table 5). Therefore, no cleaning and drying up of the interdigital space of bovine claws was necessary to obtain a suitable material for diagnosis of the foot rot bacteria.

Comparison of three different detection methods revealed the highest sensitivity of the detection of both bacteria with a cultivation of samples in AB with subsequent extraction of DNA with freezing and boiling (Table 6). The cultivation of swabbing material in anaerobic broth resulted in the detection rate of D. nodosus and F. necrophorum in over 95% and 27%, respectively. The lowest detection sensitivity could be seen when incubation of the samples was in PBS. This way of sample processing combined with DNA extraction by freezing and boiling was able to detect D. nodosus and F. necrophorum in over 61.3% and 3.75%, respectively. Fischer’s exact test revealed a significant difference in the detection rate of D. nodosus and F. necrophorum between tested extraction methods (P<.05).

When comparing the detection rate of D. nodosus and F. necrophorum in all 11 cows a significantly higher incidence (P<.05) was found for D. nodosus (100%) than for F. necrophorum (54.6%).

4. Discussion

In a Norwegian study on claw diseases (Knappe-Pointdecker et al. 2013) the majority of the tested herds were positive for D. nodosus. The presented prevalence of D. nodosus was 94.5% in problematic and 66% in control dairy herds. In the study, surface swabs were used to identify the anaerobic bacteria. The study could confirm the results of the previous observation, which found the sensitivity to be three times higher when detecting D. nodosus by PCR than by culture (Frosth et al. 2012). Similar results were obtained in cattle when the bovine skin was cleaned with tap water and dried off with...
paper towels (Knappe-Pointdecker et al. 2013). Two different sampling methods were tested in our study to select an optimal collection method for the detection of *D. nodosus* and *F. necrophorum* on the feet of dairy cows. ‘Surface’ swab sample without cleaning of the interdigital space could be associated with a detection of bacteria originating from bedding and floor. However, the same detection rate of ‘surface’ and ‘deep’ swabs for both anaerobic bacteria shows a possibility to use the ‘surface’ swab method in the veterinary practice. Our results show that there is no need for ‘deep’ swab, thus, the cows do not need to be restrained in the claw crush for sample collection. Hickford et al. (2010) selected the interdigital part of the feet as a sampling point for the detection of anaerobic bacteria. However, the samples were obtained by scraping the skin in their study. Other sampling points were chosen by Zhou et al. (2009) who made swabs at the axial part of the hoof at the transition between the skin and horn.

Swab samples were processed in the manner of the study reported by Bhat et al. (2012). The swabs they used for PCR diagnostics were incubated in PBS without EDTA after sampling. Zhou et al. (2009) and Knappe-Pointdecker et al. (2013, 2014) reported that incubation of the samples was carried out in PBS containing EDTA. Similarly, an incubation in PBS containing Na₂EDTA was used to detect *D. nodosus* and *F. necrophorum* in the study from New Zealand (Hickford et al. 2010). A prerequisite for the use of EDTA in PBS is determined by the chelating effect which is the ability to bind metal ions which may aid the tissue disruption. It is not absolutely clear whether EDTA in PBS can increase the efficiency of PCR diagnostics. It is assumed that the EDTA helps to gain a higher amount of DNA molecules during DNA extraction from the sample. The aim of our study was to investigate whether the amount of bacteria present in samples incubated in PBS is sufficient for their detection by PCR, or their numbers have to be increased by cultivation in the anaerobic broth (AB). Our results show that the anaerobic cultivation for 48 h at 37°C increases the efficiency and reliability of PCR method. The incubation in PBS only seems not be sufficient for detection of *D. nodosus* and *F. necrophorum* as a very high risk of false negative results.

In our study, the most effective method for DNA extraction was an anaerobic incubation followed by freezing and boiling. In contrast, the DNA obtained with PBS incubation method can be contaminated with proteins and other substances which can inhibit the PCR reaction. Therefore, the concentration of DNA and its possible contamination should be controlled by spectrophotometric measurement of the absorbance. DNA extraction using the isolation kit (ROCHE) led indeed to higher purity but a lower concentration of DNA in the samples. Our aim was to find out whether the extraction with the kit will increase the sensitivity of PCR, despite the reduction of the concentration of DNA (the DNA extracted using kits decreases the total yield). The results pointed to a higher percentage of positive samples in samples with a higher concentration of DNA, but with lower purity. The explanation is probably higher efficiency due to the higher purity of DNA, while in polluted samples it played a negative role with substances acting as inhibitors of PCR. Although the samples extracted with this method (incubation in PBS and extraction with the kit) were tested in a high percentage positive but were not as convincing as the results that we obtained from anaerobic cultivation (AB) and subsequent extraction by freezing and boiling. This method can cause equally polluted DNA as there is no step to remove proteins and other organic substances. However, we can assume that the concentration of DNA in the sample is in an amount in which the effect of inhibitors of the PCR is minimized. The end results depend more on the purity of the isolated DNA in PCR than on its concentration.

5. Conclusions

Both *D. nodosus* and *F. necrophorum* are common inhabitants of the skin in the interdigital space of a cattle foot. Our results showed a sufficient reliability of a single ‘surface’ swab for detection of both the anaerobic bacteria. Based on our results it can be concluded that the PCR diagnostics preceded by an anaerobic culture and DNA isolation by freezing and cooking is the most sensitive method for detection of *D. nodosus* and *F. necrophorum* on cattle foot.

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Disclosure statement

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