Molecular Identification of Clostridium chauvoei from Common Filter Paper

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ABSTRACT

Background: Blackleg is an acute and often fatal infection in bovine caused by the bacterium Clostridium chauvoei. The absence of conclusive diagnosis of blackleg usually occurs due to absence of practical and economical methods to send samples to microbiology laboratory. The goal of this work was to verify the possibility of using ordinary filter paper as a practical and economically feasible method for collecting, storing and shipping material to the laboratory to be used in a rapid and direct PCR approach to detect Clostridium chauvoei DNA.

Materials, Methods & Results: The PCR technique for the diagnosis of blackleg from common filter paper was tested for specificity, sensitivity and feasibility. To test the specificity, the papers were impregnated with a suspension of the following microorganisms: C. chauvoei, C. perfringens, C. septicum, Bacillus anthracis, Staphylococcus aureus, and Escherichia coli. To test the sensitivity different concentration of C. chauvoei (ATCC 10092) were pipetted on common filter paper. To both test, DNA extraction of impregnated ordinary filter paper and their respective controls followed the method previously described and tested under different storage times (0 h, 24 h, 72 h and a week later). To test the feasibility, 12 bovine livers were collected and tissues samples were impregnate on common filter paper with suspension of C. chauvoei. The filter paper was stored for 48 h, 72 h and one week. Subsequently, a rapid and direct PCR approach to detect C. chauvoei was performed. All procedures were performed in triplicate and was performed by PCR using the same primers employed to amplify the fl i c gene encoding flagellin (FliC). There was no cross reaction with any tested microorganism, confirming the specificity of the fl i c gene previously studied. It was possible to visualize the amplification until the corresponding to 100 CFU. Specific PCR amplification products were visualized in 100% of the trials at 48 h, 70% at 72 h, and 90% within one week of storage at room temperature using direct PCR.

Discussion: This report describes a rapid, highly sensitive method for the detection of C. chauvoei DNA from liver tissue bovine samples stored on filter papers. It was observed a high sensitivity and a specificity of 100%. The selection of hepatic tissue was based on previous studies that identified C. chauvoei in this tissue by PCR assays. Besides, blackleg in visceral form can be detected in hepatic tissue but does not in muscle. According to others researchers, the direct PCR procedure exhibits several advantages, such as costs and time reduction through omission of DNA extraction as well as avoid any cross contamination with other agents. However, current substances in the blood and tissues may inhibit the PCR amplification. For this reason, a methanol fixation and preheating the samples before the direct PCR assay was performed, mainly because the amplicon is relatively large (535 bp). Some authors consider the use of direct PCR from filter paper simple and inexpensive which offer a handy tool for epidemiologic studies and to clinicians, particularly in many tropical countries where collection and storage of clinical specimens for this purpose are logistically complicated. Furthermore, this procedure can simplify the material shipment for laboratory diagnosis, since it can also be transported in standard envelopes by regular mail. The current results propose the use of the direct PCR from common filter paper as practical and economical alternative to diagnosis of blackleg.

Keywords: blackleg, clostridiosis, Clostridium chauvoei, direct PCR, diagnosis, molecular identification.
INTRODUCTION

Blackleg is an acute and often fatal infection in cattle caused by the bacterium *Clostridium chauvoei*. It affects mainly cattle and produces high economic losses in livestock [16]. The etiologic diagnosis of infectious myonecrosis is essential for the adoption of control measures. However, the main diagnostic tests are based only on clinical findings and necropsy [1] due to difficulties to send the samples to perform the isolation and identification of the etiological agent in laboratory [12].

The polymerase chain reaction (PCR) has become possible for several laboratories to provide a faster and more accurate diagnosis by nucleic acid analyses. The DNA extraction of infectious microorganisms from filter paper impregnated with blood or tissue facilitates the shipment of samples to the laboratory; besides the feasibility of the genetic material in this media represents a great advance in the diagnosis of diseases [9]. Thus, the goal of this study was to verify the possibility of using ordinary filter paper as a practical and economically feasible method for collecting, storing and shipping material to the laboratory to be used in a rapid and direct PCR assay for molecular diagnosis of blackleg.

MATERIALS AND METHODS

To test the specificity of the PCR technique for the diagnosis of blackleg from common filter paper 1 initially, the paper was cut into small circles with an area of 3.14 cm² and they were impregnated with a suspension quantified at 10⁵ CFU (colony forming units) mL⁻¹ by plate count method of the following microorganisms: *Clostridium chauvoei* (ATCC 10092), *C. perfringens* [7], *C. septicum* (ATCC 8065), *Bacillus anthracis* (strain Stern), *Staphylococcus aureus* (ATCC 25923), and *Escherichia coli* (ATCC 25922). To test the sensitivity of this technique different concentration of *C. chauvoei* (ATCC 10092) were pipetted on common filter paper, in a volume of 50 µL containing 10⁶ to 10⁴ CFU mL⁻¹. To control any possible PCR inhibitor present in the filter paper, 50 µL of each suspension were also stored into a new 1.8 mL fresh DNAse/RNAse free microtube and tested in a *C. chauvoei*-specific PCR assay.

The DNA extraction of impregnated ordinary filter paper and their respective controls followed the method previously described [15]. All experiments were made in triplicate and tested under different storage times (0 h, 24 h, 72 h and a week later). To proceed the DNA extraction, first the impregnated filter paper and controls were incubated at 37°C for 1 h a new 1.8 mL fresh DNAse/RNAse free microtube containing 1 mL of TES buffered (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl), 25% sucrose plus 3 mg mL⁻¹ lysozyme². Subsequently, 100 µL of 10% SDS solution were added and the microtubes were incubated at 37°C for 10 min. Then, the nucleic acid was extracted with buffered phenol-chloroform and chloroform alone. The DNA precipitation was achieved with isopropyl alcohol overnight and 70% ethanol. After drying, the pellet was resuspended in 50 µL MilliQ water. The PCR was performed in a final volume of 25 µL, containing 2.5 µL of 10X buffer (100 mM Tris-HCl pH 8.5, 500 mM KCl), 5 mM MgCl₂, 40 pmol of each primer previously tested [12], 0.6 mM of each deoxynucleotide triphosphate (dNTP), 1 U Taq DNA Polymerase³, 5 µL of template DNA (approximately 50 ng) and ultra pure water up to 25 µL. All the amplifications were carried out in a programmable thermal cycle⁴ using an initial denaturation at 94°C for 2 min, 30 cycles of 1 min at 94°C for denaturation, 1 min at 57°C for annealing and 1 min at 72°C for extension and after a final extension at 72°C for 7 min and culling to 4°C. A 10 µL aliquot of PCR amplification was submitted to electrophoresis on 1.5% agarose gels, stained with ethidium bromide (0.5 mg mL⁻¹) and visualized under ultraviolet light.

To test the feasibility of *C. chauvoei* DNA from filter paper imprinted with liver tissue at different storage times, 12 bovine livers were collected into fresh sterile 15 mL graduated conical tubes from 12 animals slaughtered in an abattoir under veterinary inspection. Ten bovine hepatic tissues were selected and imprinted on common filter paper with 50 µL of suspension containing 10⁶ CFU of *C. chauvoei* on each filter paper. Two non-infected bovine hepatic tissues were imprinted on filter paper to be tested as negative controls.

A protocol formerly described [13] was adapted to perform the PCR using directly the common filter paper imprinted with bovine hepatic tissues and fixed with methanol, without a prior DNA extraction. Each common filter paper was stored for predetermined periods, including 48 h, 72 h and one week. Small portions with an area of 3.14 mm² containing 10² CFU were removed from each sample using the razor sharp
stainless steel cutting tip. Subsequently, these small portions were placed into a new 0.2 mL fresh DNase/RNase free microtube, added 5 µL of absolute methanol and dried at room temperature for 10 min. Later, in each tube was added the PCR master mix, as described above, except the Taq DNA polymerase. The tubes were heated in the programmable thermal cycler to 95°C for 10 min, and the Platinum Taq DNA polymerase was added to each microtube. The PCR cycling parameters used were 35 cycles of 30 s at 94°C for denaturation, 30 s at 57°C for annealing and extension at 72°C for 1 min. Amplified PCR products were separated by electrophoresis and visualized in 1.5% agarose gel stained with ethidium bromide (0.5 mg mL⁻¹).

All procedures were performed in triplicate. The molecular identification of *C. chauvoei* was performed by PCR using the same primers employed to amplify the *flic* gene encoding flagellin (FliC) according to prior studies [5,12].

**RESULTS**

It was possible to demonstrate the potential use of common filter paper for collection, storage and shipment of samples containing *Clostridium chauvoei* kept at room temperature to perform the laboratory diagnosis of blackleg. In this study, there was no cross reaction with any tested microorganism, confirming the specificity of the *flic* gene previously studied [5,12], even with the addition of common filter paper.

The DNA extracted from common filter paper impregnated with microorganisms assayed demonstrated to be useful since the PCR it was not inhibited and the sensitivity of the technique was not compromised. The PCR generated enough amounts of 535 bp-DNA products to be detected in an agarose gel stained with ethidium bromide. It was possible to visualize the amplification until the corresponding to 100 CFU. In a previous study [5] the PCR detection threshold was equivalent to 200 CFU.

The results obtained by direct PCR using common filter paper impregnate with bovine hepatic tissue plus *C. chauvoei* demonstrated to be feasible, and it was possible to verify amplifications in 100% of the samples at 48 h, 70% at 72 h, and 90% after a week of storage (Figure 1).

**DISCUSSION**

For the diagnosis of Blackleg should be taken into account the clinical and pathological findings added to the molecular diagnosis. The selection of hepatic tissue was based on previous studies that identified *Clostridium chauvoei* in liver tissue samples by PCR assays [2,5,6]. Besides, in visceral form as well as in classical form of blackleg is possible to detect nucleic acid of *C. chauvoei* in hepatic tissue, but muscle tissue can only be used for diagnosis of disease in the classical form [17].

According to some researchers [3,10] the direct PCR procedure exhibits several advantages, such as costs and time reduction through omission of DNA extraction as well as avoid any cross contamination with other agents. However, there are concerns about this technique, especially due to current substances in the blood and tissues that can interfere with cellular lysis and/or inactivate the thermostable DNA polymerase [9,18]. In this research, several fragments of hepatic tissue were tested to assess if there would be no interference and/or inhibition in the direct PCR. Before the direct PCR assay it is very important to perform a prior methanol fixation of the tissue already imprinted on the filter paper. According to a previously published review on the utility of PCR for DNA analysis from dried blood spots on filter paper blotters, the methanol fixation helps to prevent any PCR inhibition [9].

Ability of *C. chauvoei* to generate spores especially in long-term storage samples is another difficulty for the direct PCR technique, but in this work it was assumed that preheating the samples can circumvent this problem [4].

Some authors [8,11] consider the use of PCR from filter paper imprinted with tissues simple and inexpensive which offer a handy tool for epidemiologic studies and to clinicians who treat severe infections in...
difficult conditions. Furthermore, the rapid diagnosis of blackleg is essential to control the disease in cattle.

However, in many tropical countries collection and storage of clinical specimens for this purpose are logistically complicated [14]. Soon, this procedure can simplify the material shipment for laboratory diagnosis, since it can also be transported in standard envelopes by regular mail [11].

CONCLUSION

The current results propose the use of the direct PCR from common filter paper as practical and economical alternative to collecting, storing and shipping material to diagnosis laboratorial of blackleg. More investigations are needed, including in vivo studies to evaluate the applicability of diagnosis methods of blackleg based on the use of filter paper.

SOURCE AND MANUFACTURER

1Melitta®, Celupa Industrial Celulose e Papel Guaíba Ltda, Guaíba, RS, Brazil.
2Lizsozima, CenBiot, Porto Alegre, RS, Brazil.
3Ludwig® Taq DNA polymerase, CenBiot, Porto Alegre, RS, Brazil.
4PTC-100, MJ Research, MJ Research®, Incorporated, MA, USA.
5Harris Uni-Core®, Ted Pella, Inc., Redding, CA, USA.
6Platinum® Taq DNA Polimerase, Invitrogen®, Carlbad, CA, USA.

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REFERENCES


