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FLUORESCENT PCR ASSOCIATED WITH CAPILLARY ELECTROPHORESIS AS A DIAGNOSTIC TOOL OF BACTERIA IN SEMEN

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ABSTRACT

This study was performed in order to evaluate the detection limit of PCR with fluorescent capillary electrophoresis for *Brucella abortus* diagnosis in bovine semen. Negative bovine semen samples were artificially contaminated with *B. abortus* (10^0 to 10^7 bacteria/mL) and DNA was extracted by phenol/chloroform protocol. DNA was amplified by PCR with oligonucleotides previously described BF–5'gcgctcaggctgccgacgcaa3' (6-FAM labeled) and BR–5´accagccattgcggtcggta3' for *B. abortus*. Oligonucleotides generated DNA fragments of 193 bp. DNA fragments visualization was done under UV light at silver stained 8% poliacrylamide gel, and fluorescent capillary electrophoresis performed in an automatic DNA fragment analyzer. The detection limit of capillary electrophoresis for *B. abortus* was 10^3 bacteria/mL, while for silver stained 8% poliacrylamide gel it was 10^5 bacteria/mL. PCR with fluorescent capillary electrophoresis is a fast, efficient and highly sensitive test for DNA detection of *Brucella* in bovine semen, and itcan be an important tool for health evaluation of the herd and semen sanitary control in artificial insemination centers.

KEYWORDS: bovine semen*; Brucella abortus*; capillary electrophoresis; diagnostic fluorescent PCR.

PCR FLUORESCENTE ASSOCIADA À ELETROFORESE CAPILAR COMO FERRAMENTA DE DIAGNÓSTICO DE BACTÉRIAS NO SEMEN

RESUMO

Este estudo avaliou o limiar de detecção da técnica de PCR aliada à eletroforese capilar para diagnóstico da *Brucella abortus* em sêmen bovino. Doses inseminantes livres de patógenos foram contaminadas experimentalmente com *B. abortus* em escalas que variavam de 10^0 a 10^7 bactérias/mL e submetidas à extração de DNA pelo método de fenol/clorofórmio. A amplificação por PCR foi realizada utilizando-se oligonucleotídeos iniciadores, previamente descritos na literatura, BF–5'gcgctcaggctgccgacgcaa3' (cromóforo FAM) e BR–5'accagccattgcggtcggta3' para *B. abortus*.) Os pares de oligonucleotídeos geraram fragmentos de 193 pb. Após PCR, a visualização dos fragmentos foi realizada em gel de acrilamida 8% corada pela prata e por eletroforese capilar fluorescente em equipamento automático de análise de fragmentos de DNA. A detecção de DNA de *B. abortus* em sêmen bovino através de eletroforese capilar fluorescente foi possível a partir de concentração de 10^3 bactérias/mL, enquanto que em gel de poliacrilamida 8% o limite de detecção foi de 10^5 bactérias/mL. A eletroforese capilar demonstrou ser uma alternativa rápida, eficaz e de alta sensibilidade na detecção de DNA de *Brucella* em sêmen bovino, podendo ser uma valiosa ferramenta para a avaliação da sanidade

do rebanho e para o controle de qualidade do sêmen produzido em centrais de inseminação artificial.

PALAVRAS-CHAVE: *Brucella abortus*; diagnóstico; eletroforese capilar; PCR fluorescente; sêmen bovino.

INTRODUCTION

Brucellosis is caused by intracellular gram-negative bacteria facultative of the genus *Brucella* and continues to be a problem for humans and animals throughout the world (POESTER et al., 2002; BRICKER, 2002). It is an anthropozoonosis of great importance both for public health and for the economy of the regions where it occurs due to the high rate of abortion and infertility in infected herds (LEAL-KLEVEZAS et al., 1995; BRICKER, 2002).

Even in countries where all forms of the disease were eradicated, preventing possible re-introduction of the disease remains essential. The availability of a good diagnostic tool is crucial to the success of prevention efforts (BRICKER, 2002). The diagnosis is made primarily by means of classical procedures, which rely on growth of the infectious agent in culture, which can take weeks, or by the detection of the agent's presence by immunological methods, which have variable sensitivity and specificity (LEAL-KLEVEZAS et al., 1995; MANTEROLA et al., 2003).

In recent years, molecular diagnostic methodology based on PCR constitutes a substantial advance in the rapid detection of bacteria in different types of samples, such as *Mycobacterium tuberculosis* in milk (FIGUEIREDO et al., 2008), *Mycoplasma* spp routine cell culture (CAMARGOS et al., 2008) and *Escherichia coli* in water, feces and milk samples (VICENTE et al., 2008).

Recently, the technique of polymerase chain reaction (PCR) began to be evaluated for the detection of infectious agents in bovine semen, becoming an efficient tool and promoting a rapid and specific diagnosis (MASRI et al., 1997; HEINEMANN et al., 1999; SMITS et al., 2000; MOORE et al., 2000; MANTEROLA et al., 2003; SANTOS, 2007). Recent studies by HEINEMANN et al. (2000) and DIAS et al. (2006) demonstrated the feasibility of the diagnosis of *Leptospira* by PCR in bull semen.

The present study aimed at evaluating the use of PCR coupled with capillary electrophoresis for the detection of *Brucella abortus* in bovine semen experimentally contaminated.

MATERIAL AND METHODS

This experiment was conducted at the Laboratory of Animal Biochemistry and Molecular Biology of the Faculty of Dentistry of Araçatuba, at the Veterinary Medicine school of UNESP, Araçatuba, São Paulo, Brazil.

Brucella abortus strains used in this study were kindly provided by the Laboratory *IRFA – Química e Biotecnologia Industrial* Ltd., Porto Alegre, Rio Grande do Sul State, Brazil. The bacteria were inactivated by heat at 100 °C for 15 minutes and its concentration (2.8×10^8 bacteria/ml) was determined in the laboratory of origin by counting of bacterial cells in the dark field.

Bovine semen samples, used for experimental infection with *Brucella*, were obtained from a Nellore bull from the *Central de Inseminação Artificial Lagoa da Serra*, in the municipality of Sertaozinho, São Paulo State. To use semen, we adopted the criteria of not presenting positive result for the agent, determined by serology or culture. We carried out the spermatozoa count in the ejaculate in a Neubauer chamber and we diluted the insemination dose in sterile saline (0.9%) for the volume of 500µL containing $3x10^7$ spermatozoa / mL.

The experimental contamination of the semen was performed with decreasing concentrations of *Brucella abortus* obtained by serial dilutions on base 10 (10^7 to 10^0 bacteria/mL), to thereby determine the lowest concentration of bacterial DNA that can be detected by the PCR technique.

We extract DNA from the bacteria used for semen experimental contamination in 1.5 mL polypropylene microtubes according to the protocol described by HEINEMANN et al. (2000). We used the following primers to amplify 193 bp of *Brucella abortus*: bf-5'GCGCTCAGGCTGCCGACGCAA3' (marked with FAM fluorescent substance - Invitrogen-Life Technologies®) and br-5'ACCAGCCATTGCGGTCGGTA3' previously described (LEAL-KLEVEZAS, et al., 1995).

We performed the PCR reactions for *Brucella abortus* detection in 200µL microtubes, in a total volume of 50 µL containing the following reagents: 5 µL of 10x reaction buffer (final concentration 200 mM Tris-HCl, pH 8.4), 4 µL of magnesium chloride (variable concentration) (Invitrogen-Life Technologies®); 2 µL of deoxyribonucleoside triphosphates (dNTPs), 1.25 mM each [dCTP, dATP, dGTP, dTTP]; 3 µL of each primer (10 pmol per µL); 1 µL of Taq DNA polymerase (Invitrogen-Life Technologies®); 5µl of DNA sample and ultrapure water qs (Invitrogen-Life Technologies®) to complete the total volume. Furthermore, we used negative controls containing all reagents except DNA for monitoring possible contamination. We submitted the samples to an initial denaturation for 5 minutes at 95 °C, 35 cycles at 95 °C for 60 seconds, 60 °C for 60 seconds, and 72 °C for 60 ºC and final extension at 72 °C for 5 minutes. Amplification conditions were carried out in thermocycler (PTC-100 MJ-Research®). To verify the species-specific amplification of the primers used, PCR was performed with DNA from semen samples experimentally contaminated with *Brucella abortus, Leptospira interrogans sorotipo pomona, Campilobacter fetus* and *Haemophilus somnus.*

We performed the analysis of bacterial DNA fragments by conventional electrophoresis in acrylamide-bis-acrylamide gel 8% (Invitrogen) stained with silver and by capillary electrophoresis equipment for automatic analysis of DNA fragments (ABI-310 Applied Biosystem) (DIAS e al., 2006).

RESULTS AND DISCUSSION

In this study, we used information of the outer membrane protein gene (omp-2) of *Brucella abortus* drawn from the classic papear by LEAL-KLEVEZAS et al. (1995), who were able to amplify *Brucella* spp. from various biological materials.

The results of DNA amplification, by PCR technique, of the semen samples experimentally contaminated with *B. abortus* showed that the primers employed resulted in specific amplifications for *Brucella* DNA (Figure 2) with the amplification of a fragment of 193 base pairs.

Specific primers (Figure 1) were used in this study in order to avoid cross-reaction with the DNA of other bactéria likely to be found in this type of biological material.

Figures 2 and 3 show the products of PCR amplification from the DNA of *B. abortus* in samples of experimentally contaminated bovine semen, where we observed the results of the amplification in polyacrylamide gel 8% and by capillary electrophoresis, respectively.

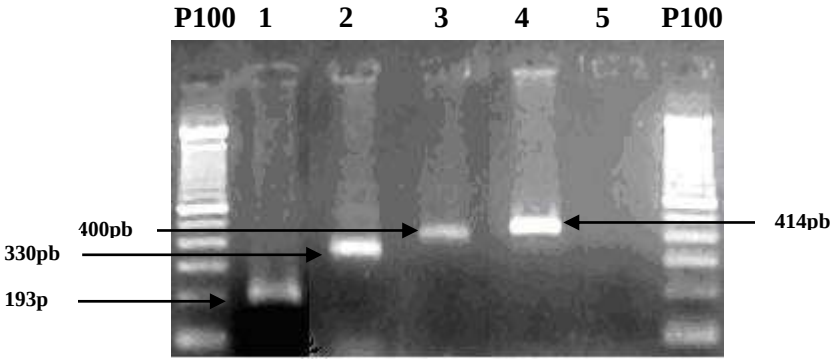

FIGURE 1 - Electrophoresis in agarose gel at 2% stained with ethidium bromide showing the specificity of the primers. (P100) Molecular weight marker in 100-bp ladder, (1-4) Products of the PCR amplification of DNA from semen experimentally contaminated with *Brucella abortus, Leptospira interrogans sorotipo pomona, Campilobacter fetus* and *Haemophilus somnus* and amplified with oligonucleotides specific for *Brucella* sp (1), *Leptospira* sp (2), *Haemophilus somnus* (3), *Campylobacter* sp (4), (5) Negative control (without DNA).

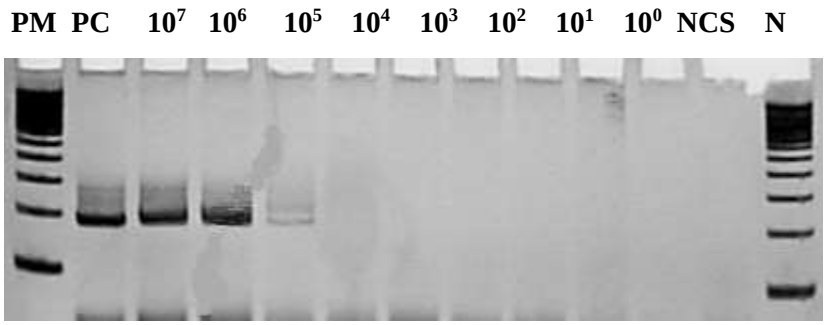

FIGURE 2 - Electrophoresis in polyacrylamide gel 8% stained with silver nitrate. Products of the PCR amplification of DNA from bovine semen experimentally contaminated with *Brucella abortus*, (PM) 100-bp molecular marker, (PC) positive control *Brucella abortus*, (10^7 to 10^0) serial dilution on base 10 bacteria/mL from the initial concentrations (2.8 x 10^8 bacteria/mL) of the *Brucella abortus* stock solution, (NCS) non-contaminated semen, (N) negative control (without DNA).

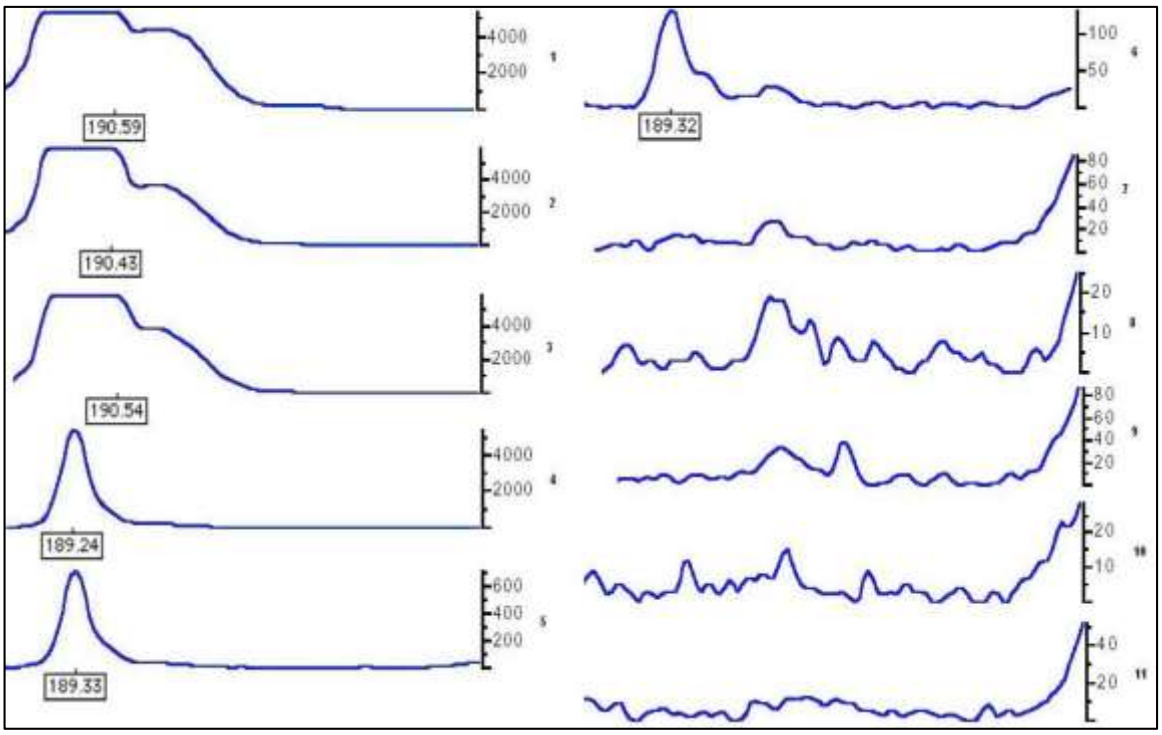

FIGURE 3 - Electropherogram with the products of the PCR amplification of DNA of bovine semen samples experimentally contaminated with *Brucella abortus*, (1) positive control *Brucella abortus*, (2 to 9) serial dilutions on base 10 (10^7 to 10^0 bacteria/mL) from initial concentrations (2.8 x 10^8 bacteria/mL) of the *Brucella abortus* stock solution, (10) non-contaminated semen, (11) negative control (without DNA).

The DNA detection of pathogenic bacteria after PCR reaction are usually performed by gel electrophoresis; however, the gel is usually little sensitive and requires a long time for separation, which limits the overall efficiency of PCR for rapid and specific analysis (SONG et al., 2003). In this study, we could verify that the analysis of DNA fragments after the extraction is faster when an automated equipment is used instead of the conventional electrophoresis, which requires more time and presents dubious results.

Figure 2 shows the threshold of *Brucella abortus* detection in bovine semen experimentally contaminated and analyzed in conventional electrophoresis on polyacrylamide gel stained with silver nitrate, indicating positivity up to 10^5 bacteria/mL from the initial concentration of the stock solution of *Brucella abortus.* When analyzed by capillary electrophoresis (Figure 3), the same sample showed a positive signal up to a dilution of 10^3 bacteria/mL. According to SONG et al. (2003), speed and sensitivity are the most important factors to be considered in the detection of bacteria, because even a single pathogenic organism may result in an infecting dose.

The use of fluorescent marking in this study coupled with the PCR detection in automatic analysis of DNA fragments can be an alternative for increasing this application (ENGLUND et al., 2001; CHECA et al., 2002; SONG et al., 2003). Few studies associate the pathogen PCR amplification with its detection in automated system for analysis of DNA fragments marked with fluorochromes. ENGLUND et al. (2001) compared the detection of *Mycobacterirum avium* subspecies *paratuberculosis* in birds biological samples using simple PCR, fluorescent PCR and nested PCR, and reported that fluorescent PCR constitutes an useful alternative to both simple and nested PCR for pathogen detection due to higher sensitivity and capacity afforded by automation.

The analysis of fluorescent PCR fragments by capillary electrophoresis in the diagnosis of infectious diseases can be considered little studied; however, we observed the best detection capacity by this method throughout the experiments in this study as well as DIAS et al., (2006), who showed that detection is enhanced when compared to conventional electrophoretic analysis on polyacrylamide gel in the detection of *Leptospira pomona* in bovine semen. Furthermore, it is noteworthy that results reading is easier in capillary electrophoresis than in polyacrylamide. These findings confirm the conclusions by ENGLUND et al. (2001), who advocated for the detection of PCR fragments with fluorescent marking not only because of the increase in sensitivity, but mainly because of the possibility of interpreting ambiguous PCR results in the electrophoresis analysis on polyacrylamide and agarose gel.

Among the various pathogens listed by the World Organisation for Animal Health (OIE) that affect cattle (THIBIER & GUERIN, 2000), some have a relevant role in Brazilian current situation, e.g. *Brucella abortus* (POSTER et al., 2002), *Leptospira* sp. (HEINEMANN et al., 2000) and *Campylobacter fetus* (VARGAS et al., 2003).

Currently, the diagnostic methods used to identify animals carrying infectious agents include isolation in chemically defined culture medium or in cell culture, inoculation of susceptible animals, serum neutralization, complement fixation test, ELISA, indirect immunofluorescence, hemagglutination and immunodiffusion (CICERONI et al., 2002; MANTEROLA et al., 2003). However, most of these techniques present practical limitations due to complexity, slowness of laboratory procedures for the detection and characterization of the infectious agent or required infrastructure for their performance. Furthermore, limitations on the sensitivity and specificity make it more difficult to achieve a practical, accurate and low-cot diagnosis (VELOSO et al., 2000; CICERONI et al., 2002; MANTEROLA et al., 2003).

According to SANTURDE et al. (1996), ROCHA et al. (1998), SMITS et al. (2000), ORTEGA-MORA et al. (2003), MUKHUFHI et al. (2003) and SANTOS (2007), technological advances in diagnostic methods, particularly those involving routine molecular biology, have a great potential to integrate screening and control tests of semen-donor bulls regarding the presence of infectious agents. In the present study, we sought to develop a methodology to access the presence of *Brucella*, which has health and hence economic relevance, in samples of bovine semen combining speed and efficiency.

Recently, PCR techniques for detection of infectious agents have been widely used due to its accuracy and sensitivity. Such advantages enable highly specific identifications, thus allowing to distinguish different bacteria serotypes (HEINEMANN et al., 1999; 2000; VARGAS et al, 2003).

By analyzing semen samples, AMIN et al. (2001) reported the application of PCR technique in the detection of *Brucella militensis* in goat sêmen, and MANTEROLA et al. (2003) evaluated PCR for the successful detection of *Brucella ovis* in ovine semen. However, there are few studies on the detection of infectious agents of venereal transmission in semen samples.

These results indicate that capillary electrophoresis can be a rapid and effective alternative for the detection of *Brucella abortus* DNA when compared to the traditional electrophoresis system. Therefore, we concluded that the capillary electrophoresis coupled with PCR is a valuable tool in the detection of *Brucella abortus* by allowing more speed and sensitivity, advantages that may, in future, be added to the conventional methods for detection of bacteria in bovine semen, becoming one more diagnosis resource to ensure the production

of semen free of infectious agents in artificial insemination centers.

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