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Detection of *Leptospira* spp. from pure cultures and from experimentally contaminated bovine semen by polymerase chain reaction

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Detecção de *Leptospira* spp. através da reação em cadeia pela polimerase (PCR) em sêmen bovino experimentalmente contaminado

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SUMMARY

Due to the high importance of the venereal transmission of bovine leptospirosis, this study aimed to test the ability of PCR to detect *Leptospira interrogans* serovar hardjo DNA in experimentally contaminated bovine semen. Employing primers directed to the 16S rRNA gene, 10 bacteria/ml of semen could be detected by PCR. Results achieved in this work show that PCR can have a great potential to detect *Leptospira* spp. in insemination centers.

UNITERMS: Leptospirosis; Leptospira; Semen; Polimerase Chain Reaction; Bovine.

INTRODUCTION

Artificial insemination is an important procedure for increasing animal production and millions of doses of frozen bovine semen are exchanged annually among different countries. To obtain high fertility and birth rates and to prevent infectious diseases, many tests are employed for the detection of infectious agents in bovine semen. Bovine leptospirosis is one of the important infectious diseases that can be transmitted by artificial insemination since *Leptospira* spp. can survive at the freezing temperatures needed to store semen[1].

Direct diagnostic methods based on leptospira isolation from semen are not easily performed due to the rapid growth of other microorganisms[8]. In addition, the absence of detectable levels of serum antibodies in infected animals makes the indirect methods inefficient[2].

Among the DNA-based techniques, the polymerase chain reaction (PCR) has been used for the diagnosis of slowly growing or fastidious microorganisms[1]. As to bovine leptospirosis, some investigators have used PCR to detect *Leptospira* spp. in bovine urine[4,6,10], and recently in bovine semen[5].

Employing primers directed to the *Leptospira interrogans* serovar canicola strain Moulton 16S rRNA gene[6], this study aimed to test the ability of PCR for the detection of several *Leptospira* spp. from pure cultures and from bovine semen experimentally contaminated with *Leptospira interrogans* serovar hardjo.

MATERIAL AND METHOD

Leptospira serovars and growth conditions

The *Leptospira* serovars used in this study (Tab.1) were cultivated at 28°C in EMJH medium supplemented with inactivated rabbit serum as described by Turner[9], and belong to the collection of the Laboratory of Bacterial Zoonoses of the Faculty of Veterinary Medicine and Zootechny, University of São Paulo - SP- Brazil.

Experimental contamination of bovine semen

The semen for experimental contamination with leptospires was collected from a bull that presented no detectable antibodies against any of the bacteria listed in Tab.1 as assessed by the microscopic agglutination test[3], and was also free of leptospires, as determined by microbiological culture. The bovine semen described above was diluted with distilled water in order to obtain 50 x 10^6 spermatozoa/ml and contaminated with 10^1 to 10^6 *Leptospira interrogans* serovar *hardjo*/ml.

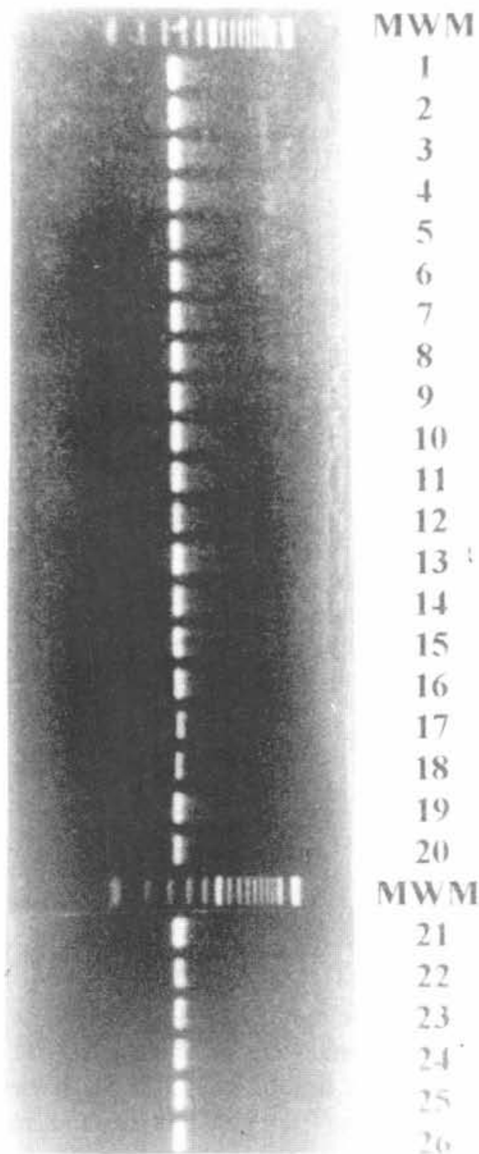

Figure 1
Gel electrophoresis performed on 2% agarose of PCR-amplified products from 26 Leptospira serovars. Lanes 1= australis; 2= autumnalis; 3= butembo; 4= castellonis; 5= bataviae; 6= brasiliensis; 7= canicola; 8= whitcombi; 9= cynopteri; 10= hebdomadis; 11= copenhageni; 12= icterohaemorrhagiae; 13= grippotyphosa; 14= javanica; 15= panama; 16= pomona; 17= pyrogenes; 18= hardjo; 19= wolffi; 20= shermani; 21= tarassovi; 22= andamana; 23= buenos aires; 24= garcia; 25= rufino and 26= patoc. A 330 bp amplified DNA fragment can be observed in all serovars. MWM= molecular weight markers ranging from 100 to 1,500 bp at 100 bp increments (Gibco-BRL Gaythersburg, MD-USA). The bands were visualized by ethidium bromide staining under ultraviolet light.

Microbiological culture

For microbiological culture 1 ml of the bovine semen contaminated with different bacterial concentrations was seeded in EMJH medium supplemented with inactivated rabbit serum, incubated at 28ºC and observed for 8 weeks.

PCR

Leptospires from experimentally contaminated bovine semen (serovar hardjo) or from pure cultures of all serovars listed in Tab. 1 were collected by centrifugation at 13,000 x g for 30 minutes at 4ºC and DNA was extracted as described by Sambrook *et al.*[7].

The primers employed were those described by Mérien *et al.*[6] corresponding to nucleotides 38 to 57

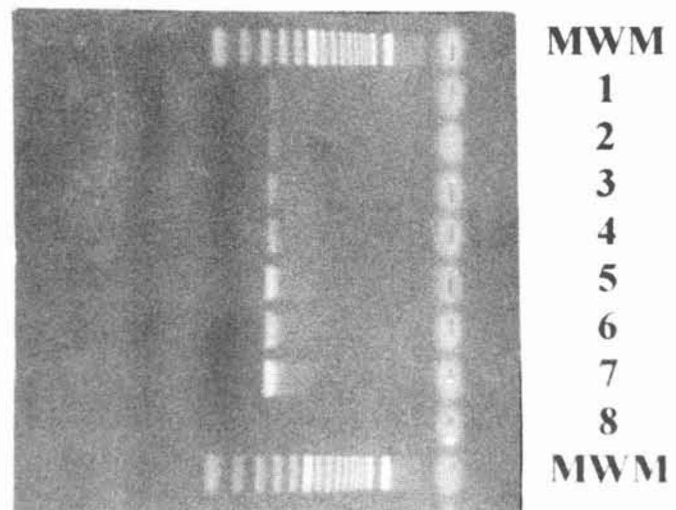

Figure 2
Gel electrophoresis performed on 2% agarose showing the threshold of detection of L. interrogans by PCR in bovine semen. Lanes 1 to 7 contain the following concentrations of Leptospira interrogans serovar hardjo: 1= 10 bacteria/ml; 2= 100 bacteria/ml; 3= 1,000 bacteria/ml; 4= 10,000 bacteria/ml; 5= 100,000 bacteria/ml; 6= 1,000,000 bacteria/ml; 7= 10,000,000 bacteria/ml. Lane 8 is the PCR control containing uncontaminated semen. MWM= molecular weight marker ranging from 100 to 1,500 bp at 100 bp increments (Gibco-BRL Gaythersburg, MD-USA). The bands were visualized by ethidium bromide staining under ultraviolet light.

(5'GGCGGCGCGTCTTAAACATG3') and 348 to 368 (5'TCCCCCCATTGAGCAAGATT3') of the 16S rRNA gene of *L. interrogans* serovar canicola strain Moulton.

DNA amplification was performed in a total volume of 50 µl. The reaction mixture consisted of 200 mM Tris-HCl, pH 8.0, 500 mM KCl, 50 mM $MgCl_2$, 1.25 mM each dATP, dTTP, dCTP, dGTP, 6.25 µM each oligonucleotide primer, 2 units of *Taq* DNA polymerase (Gibco BRL Gaythersburg, MD-USA) and the sample, comprised in 1/10 of the final volume. The reaction mixture was overlaid with 50 µl of mineral oil (Sigma). PCR was performed as described by Mérien *et al.*[6] using a Perkin Elmer 480 thermal cycler.

The amplification products were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide and visualized under UV light.

The threshold of *Leptospira* detection by PCR and by microbiological culture

The threshold of *Leptospira* detection by PCR and by microbiological culture was evaluated in the experimentally contaminated bovine semen.

RESULTS AND DISCUSSION

Fig. 1 shows that PCR amplified a 330 bp DNA fragment from the DNA of all *Leptospira interrogans, Leptospira borgpetersenii, Leptospira noguchii, Leptospira santarosai* and *Leptospira biflexa* serovars used in this study (Tab. 1).

The different papers that describes PCR assays for the detection of leptospires in bovine urine[4,10] or semen[5] were not able to identify specific serovars, since the primers amplified conserved regions of *Leptospira* spp. genome.

Recently Masri *et al.*[5] described a PCR followed by a nested PCR assay to detect *Leptospira* spp. in bovine semen experimentally contaminated with the serovar hardjobovis. The primers employed by these authors were derived from a 3,800 bp EcoR1/BamH1 fragment of serovar hardjobovis genome and hybridized to 11 of 31 not specified serovars.

The broad spectrum of PCR in the detection of pathogenic and saprophytic leptospires, also observed in the present study, could be explained by the primers employed since the sequence of *Leptospira* 16S rRNA gene is highly conserved[6].

In addition to the *Leptospira* serovars originally detected

Table 1

Leptospira serovars used for PCR analysis. São Paulo, 1996.

Species	Serogroup	Serovar
L. interrogans	AUSTRALIS	australis
L. interrogans	AUTUMNALIS	autumnalis
L. interrogans	BUTEMBO	butembo
L. borgpetersenii	BALLUM	caslellonis
L. interrogans	BATAVIAE	bataviae
L. santarosai	BATAVIAE	brasiliensis
L. interrogans	CANICOLA	canicola
L. borgpetersenii	CELLEDONI	whitcombi
L. interrogans	CYNOPTERI	cynopteri
L. interrogans	HEBDOMADIS	hebdomadis
L. interrogans	ICTEROHAEMORRHAGIAE	copenhageni
L. interrogans	ICTEROHAEMORRHAGIAE	icterohaemorrhagiae
L. interrogans	GRIPPOTYPHOSA	grippotyphosa
L. borgpetersenii	JAVANICA	javanica
L. noguchii	PANAMA	panama
L. interrogans	POMONA	pomona
L. interrogans	PYROGENES	pyrogenes
L. interrogans	SEJROE	hardjo
L. interrogans	SEJROE	wolffi
L. santarosai	SHERMANI	shermani
L. borgpetersenii	TARASSOVI	tarassovi
L. biflexa	ANDAMANA	andamana
L. biflexa	ANDAMANA	buenos aires
L. biflexa	GARCIA	garcia
L. biflexa	RUFINO	rufino
L. biflexa	SEMARANGA	patoc

by PCR by Mérien *et al.*[6], the following serovars were detected in the present study: andamana, brasiliensis, buenos aires, butembo, garcia, rufino and whitcombi.

Employing PCR and nested-PCR Masri *et al.*[5] detected respectively 500 - 5,000 and 50 - 500 organisms/ml of semen. The PCR assay here described detected 10 *Leptospira interrogans* serovar hardjo/ml in bovine semen (Fig. 2), a threshold not reached by microbiological culture that needs a minimum of 1,000 bacteria/ml.

Among the different factors that could explain the lower threshold obtained in this study were: 1) The DNA extraction protocol that involved procedures used in the 3 different protocols evaluated by Masri *et al.*[5]; 2) The reagents, primers and DNA amplification conditions.

In addition to a lower detection limit, PCR has the advantage of being performed within a shorter period of time than *Leptospira* culture, which can take up to 2 months[3].

Considering serology does not reflect the carrier or shedding status of cattle[2] and the difficulties of *Leptospira* isolation from bovine semen, PCR may represent a useful tool for the rapid detection of *Leptospira* spp. in bovine semen at artificial insemination centers.

RESUMO

Considerando a importância do sêmen na transmissão da leptospira bovina, foi realizado o presente estudo que teve como objetivo aplicar a reação em cadeia pela polimerase (PCR) para a detecção de leptospiras em sêmen bovino experimentalmente contaminado. A reação de PCR foi capaz de amplificar um fragmento de DNA específico de 330 pares de bases a partir de cultivos puros de 26 sorovares de *Leptospira* spp. A contaminação experimental de sêmen com *Leptospira interrogans* serovar hardjo revelou que a técnica de PCR conseguiu detectar 10 bactérias/ml, concentração sensivelmente mais baixa que as 1.000 bactérias/ml detectadas através do cultivo microbiológico. Os resultados observados revelam o grande potencial da reação de PCR para a detecção de *Leptospira* spp. em sêmen bovino, notadamente em centrais de inseminação artificial.

UNITERMOS: Leptospirose; Leptospira; Sêmen; Reação em cadeia de polimerase; Bovinos.

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