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Animal Reproduction Research Institute
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RAPID DETECTION AND DIFFERENTIATION OF LEPTOSPIRA BY POLYMERASE CHAIN REACTION

(With 4 Figures)

By

G.S. RADWAN, E.R.H. ATTIA and M.S. TAWFIK

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الكشف السريع عن والتفريق بين ميكروب وعترات الليبتوسبيرا باستخدام اختبار ال بى. سى .أر.

جمال رضوان ، السيد رضوان عطيه ، محمود توفيق

تم استخدام اختبار ال بى . سى . أر. للتفريق بين عترات الليبتوسبيرا . وقد أمكن باستخدام هذا الاختبار التفريق السريع لعترات مرجعيه أختبرت من الليبتوسبيرا هاردجو وبومونا وكانيكولا. وفى تجربة أخرى مكن هذا الاختبار من الكشف السريع بنجاح عن الحمض النووى المميز للميكروب فى بول الأبقار المحقون معمليا بعترة الليبتوسبيرا هاردجو . وقد تم التأكد من تخصص الاختبار فى الكشف عن الحمض النووى الخاص بالميكروب فى عينات البول عن طريق أجراء اختبار التهجين مع مجس الحمض النووى الديوكسى ريبوزى غير المشع المحضر من الليبتوسبيرا هاردجو . وقد دلت النتائج على أنه باستخدام اختبار ال بى . سى . أر . يمكن التفريق والتصنيف السريع لعترات ومعزولات الليبتوسبيرا وإمكانية تطبيقه كاختبار ذو قيمة فى الكشف بتخصص عن ميكروبات الليبتوسبيرا فى العينات الحقلية من الأبقار :.

SUMMARY

The technique of arbitrarily primed polymerase chain reaction (AP-PCR) or random amplified polymorphic DNA (RAPD) fingerprinting was used for differentiation of leptospira. The assay allowed the rapid differentiation of standard: *L.hardjo, L.pomona and L.canicola* examined reference strains. In another experiment, this PCR assay successfully detected leptospiral DNA in *L.hardjo*-seeded bovine urine. The specificity of leptospiral DNA-amplification from urine samples was confirmed by southern blot hybirdization using a nonradioactive labeled DNA probe. The data suggested that AP-PCR may be used for rapid differentiation and typing of leptospiral

isolates, and a valuable test for specific detection of leptospira in bovine field samples.

Key words: *Leptospira - Detection - PCR*

INTRODUCTION

Leptospirosis is a common zoonotic disease affecting most mammals with world - wide distribution. In live-stock, the disease causes high economic losses due to abortion, stillbirths, infertility, decreased milk yield and death at young ages (Ellis et al., 1985) . For diagnosis of leptospirosis, a rapid and specific assay is needed where an infection can be detected and treated at early stage . Because the organism persists in the kidneys and genitals without clinical signs of disease (Ellis et al., 1986), carrier cows often excrete leptospira in their urine, such cattle are an important source of infection not only for other cows, but also for dairy farm workers and other people (Waitkins, 1986).

The detection and characterization of leptospira are currently done with culture isolation techniques. However, culture is slow, laborious and susceptible to contamination . DNA-based techniques has been introduced into the field of leptospirosis. Assays such as restriction endonuclease analysis and southern blotting may be suitable for strain identification but they are time-consuming and laborious (Thiermann et al., 1986 and Van Eys et al.,1988). More recently, Welsh and McClelland (1990) and Williams et al. (1990) described a rapid random amplified polymorphic DNA (RAPD) fingerprinting technique based on arbitrarily primed amplification of genomic sequences by using a single primer in a polymerase chain reaction (PCR). The objective of this paper was to investigate the use of RAPD fingerprinting (AP-PCR) for rapid differentiation of leptospiral strains. In addition, the application of this PCR assay for detecting leptospiral DNA in seeded bovine urine was also studied.

MATERIALS and METHODS

Leptospiral standard strains

L.hardjo , *L. pomona* and *L.canicola* reference strains (kindly provided by Dr. Cathrine Sulzer, the Center for Diseases Control, Atlanta,GA, USA) were used in this study . The strains were maintained on EMJH media.

Urine samples

Midstream urine samples were aseptically collected from cows . A one ml volume of *L.hardjo* culture ($1x10^7$ cells /ml) was added to 9 ml of the urine sample. leptospira negative control urine samples were also tested.

Preparation of leptospiral DNA

DNA was prepared for PCR as described by Woodward and Redstone (1993). Briefly, leptospira from 10 ml of young cultures (5 - 7 days old) or the seeded cattle urine were collected by centrifugation at 13,000 X g for 15 minutes at 4°C. Cellular pellets were resuspended in 0.1 ml of TE buffer (Tris 10 mM, EDTA 1mM; pH 7.5). DNA was extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform :isoamyl alcohol(24:1) and then ethanol precipitated.

DNA preparations were run through 1% (W/V) agarose in TAE buffer (0.04 M Tris-acetate, 0.01M EDTA), stained with ethidium-bromide (0.5µg/ml) and visualized by using ultraviolet transillumination according to the methods of Sambrook et al.(1989).The final DNA concentration of each sample was determined by spectrophotometry. The DNA samples were then either stored at -20°C or directly subjected to PCR .

PCR

One 19-mer primer (L1; 5' GTA GAG CTC GCG GCA CTT G 3') and one 17 mer primer (M13 universal sequencing primer, L2; 5' GTA AAA CGA CGG CCA GT 3') were used (Corney et al., 1993). Primers were obtained from Agricultural Genetic Engineering Research Institute, ARC, Giza). PCR mixtures contained 1X PCR buffer, 200 µM (each) deoxynucleoside triphosphate, one of the above primers at 50 nM, 4 mM magnesium chloride, 2.5 U of Taq polymerase (Promega Corp., Madison, WI, USA) and 70 ng of purified DNA in a 100-µL reaction volume. The temperature program consisted of 1 cycle with 3 minutes at 97 °C, 1 minute at 40 °C, and 1 minute at 72 °C; 4 cycles with 1 minute at 97 °C, 1 minute at 40 °C, and 1 minute at 72 °C; 24 cycles with 1 minute at 95 °C, 1 minute at 55°C, and 1 minute at 72 °C; and 1 cycle with 1 minute at 95°C,1 minute at 55 °C, and 7 minutes at 72°C. The temperature program was run in an 110 P thermal cycler (Coy Corp., Grass Lake, MI, USA). A negative control PCR mixture with no template also was included in the assay. Reaction products were analyzed on agarose as previously described and photographed over the UV light source.

Alkali blotting and DNA hybridization

Following electrophoresis, PCR products from urine samples were transferred to Nylon membrane (Sigma, St. Louis, MO, USA) by a TE 80 vacuum blotter (Hoefer, San Francisco, CA, USA) in 0.4 N Na OH (Southern, 1975). *L.hardjo* DNA was labeled with digoxigenin - 11 - d UTP (Boehringer Mannheim Corp., Indianapolis, IN, USA) by random priming and hybridized to the nylon membrane at 68°C. Labeling, hybridization and detection were performed according to the manufacture's instructions

RESULTS

RAPD profiling of leptospiral reference strains

To establish a rapid differentiation system based on RAPD fingerprinting, we prepared RAPD profiles for the tested reference *strains: L.hardjo, L. pomona and L. canicola.* Purified DNA from these strains (Fig.1) was amplified with primer L1 and L2 (Fig. 2). These profiles, containing a number of DNA bands, were generated when the PCR reaction products were run on the agarose gel. Arrowheads on that figure indicate features useful for differentiation of the tested strains. The negative control reaction sample (with no template) gave a negative PCR result (Lane 8).

PCR analysis of bovine urine samples

The results of PCR assay on DNA obtained from bovine urine samples are given in Fig.3. The PCR assay using the L1 primer detected leptospiral DNA extracted from *L.hardjo*-seeded bovine urine samples (Lane 2) The PCR profile obtained from urine sample was identical to that of the *L. hardjo* reference culture (Fig. 2 , Lane 4). No detectable product DNA was observed following PCR of negative control urine sample (Fig.3, Lane3). In order to confirm the specificity of leptospiral DNA amplification from urine samples, a hybridization assay using a nonradioactive leptospira-specific probe was conducted. Fig. 4 shows the result of Southern blot hybridization of PCR products from urine samples and the *L. hardjo* digoxygenin - 11 - dUTP labeled DNA probe. The probe reacted specifically with PCR product DNA from the *L.hardjo* seeded bovine urine sample (Lane 1) . On cotrast, no hybridization signal was observed for PCR product from negative control urine sample (Lane 2).

DISCUSSION

Accurate identification of leptospiral species or strains is very important in the diagnostics and epidemiological studies of the disease. Current methods available for the detection and characterization of leptospira such as culture isolation and serological techniques lack rapidity or specificity for consistent testing (Ellis et al., 1982 ; Thiermann , 1983 and 1984).

Developments in molecular biology techniques and molecular characterization of leptospira have provided methods for detection and identification that have the potential to be very rapid specific (Le Febvre , 1987 ; Terpstra et al ., 1987 ; Bolin et al., 1989 ; Nielsen et al., 1989) . The availability of nucleotide sequences permitted the development of the PCR for the rapid detection and differentiation of leptospira (Van Eys et al., 1989 ; Gerritsen et al., 1991; Woodward and Redstone , 1993).

In this study, the technique of RAPD or AP- PCR (Welsh and McClelland, 1990 ; Williams et al.,1990) was used as simple and rapid procedure to detect polymorphism in leptospiral strains. RAPD (AP- PCR) is distinct from the standard PCR because it involves random amplification of genomic DNA with single primers of arbitrary sequence. This fingerprinting assay has been used to differentiate and type brucella (Fekete et al., 1992) and campylobacter (Mazurier et al., 1992) strains. Our results show that RAPD fingerprinting allows the rapid differentiation of leptospiral strains, as characteristic profiles were generated for each strain . In our RAPD assay, a negative control with no template DNA was introduced into the assay. This was done in order to eliminate the risk of obtaining false positive results due to possible carryover contamination between tested samples (Kwok and Higuchi, 1989). The negative PCR result on that sample ruled out this probability. With the use of both L1 and L2 primers, the tested reference leptospiral strains were distinctly identified. Our data suggest that primers L1 is more discriminatory than L2 for differentiating the tested strains. Thus, L1 appears the more useful of the two primers used in this assay. Similar results were reported by Corney et al. (1993). This RAPD fingerprinting technique can be used as a rapid method for identification of leptospira. Profiles generated from field isolates could be compared with those generated from reference strains for their fast identification and typing. An isolate could be typed in as little as one working day. Rapid and simple identification of such

isolates would help in rapid gathering and assessment of epidemiological data to allow the institution of appropriate control measures. Although culture techniques can be used to detect leptospira in urine, they are labor intensive and slow that require weeks or months before results are obtained (Thiermann, 1984).Therefore, the application of PCR as an alternative diagnostic assay for detecting leptospiral DNA in leptospira-seeded bovine urine was investigated in this study. DNA extraction from urine is cumbersome, mainly because of the presence of PCR inhibitors (Gerritsen et al.,1991). The sample preparation and DNA extraction method described in this study enables us to use the PCR to reliably detect *L hardjo* in bovine urine.The PCR profile obtained from *L.hardjo*-seeded bovine urine samples using primer L1 was identical to that generated from reference strain culture. This suggests the specificity of PCR amplification of leptospiral DNA and the success of using PCR assay for detection of leptospiral specific DNA in urine samples. In order to detect any false-positive results, a negative control for each urine sample was introduced in the assay as each urine sample was divided into two 9- ml portions and one of them was seeded with *L.hardjo* cells. Results of the PCR were considered reliable because the leptospira-seeded and negative control urine samples gave positive and negative results on PCR assay respectively. Moreover, the negative PCR result of negative control samples excluded the possibility of amplification of DNA from nonleptospiral microorganisms that might contained in bovine urine. The specificity of PCR amplification for leptospiral DNA specific detection in bovine urine samples was further confirmed by Southern blot hybridization with *L.hardjo* nonradioactive labeled DNA probe. Hybridization of the Southern blot of the electrophoresed PCR products from *L. hardjo*-seeded urine sample confirmed that the amplified fragments were complementary with *L. hardjo* sequences. In this work, we have shown that AP-PCR assay may provide a useful additional and alternative method for characterization of leptospiral strains. In addition, the use of PCR assay with its speed and specificity can rapidly and specifically detect leptospiral DNA in bovine urine. The data suggest that the PCR assay can be used as a novel tool for diagnostics as well as epidemiological studies of leptospirosis.

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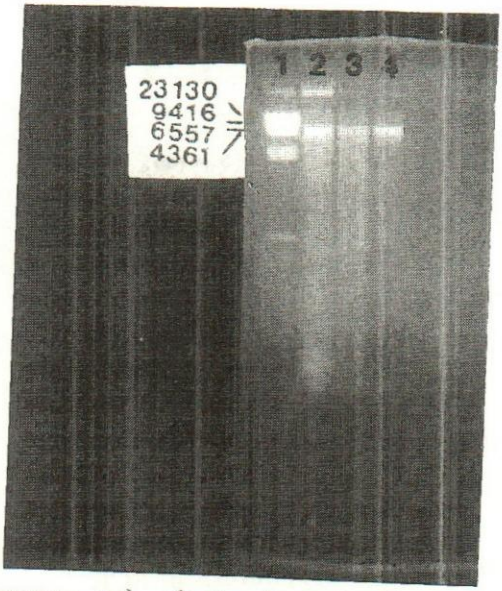

Fig. 1 : Agarose gel electrophoresis of genomic DNA's extracted from standard leptospiral reference strains. Lane 1 , λDNA Hind III molecular weight marker in base pairs ; lane 2 , *L. hardjo*; lane 3 , *L. pomona;* lane 4 , *L canicola.*

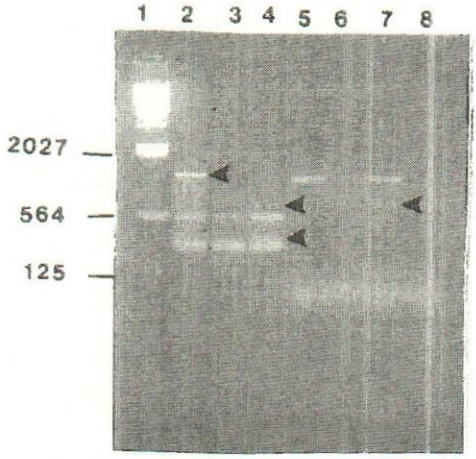

Fig. 2 : RAPD profiles of the leptospiral strains with L1 (lanes 2 , 3 and 4) and L2 (lanes 5 , 6 and 7) primers Lane 1, molecular weight marker in base pairs; lane 2 and 5, *L. pomona* ; lanes 3 and 6 , *L. canicola* ; lanes 4 and 7, *L. hardjo* ; lane 8, negative control reaction without template DNA. Arrowheads indicate features useful for differentiation of the tested strains.

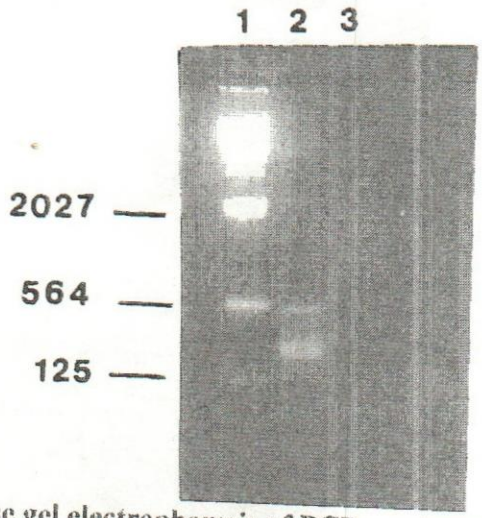

Fig. 3 : Agarose gel electrophoresis of PCR products from bovine urine samples using the L1 primer. lane 1, molecular weight marker in base pairs ; lane 2 , urine sample seeded with *L.hardjo* cells; lane 3 , urine samples with no leptospira added (negative control) .

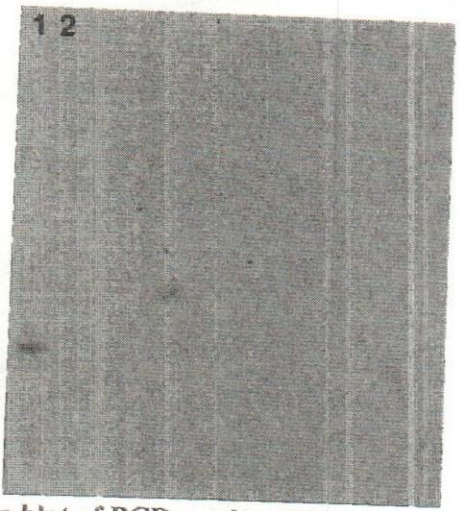

Fig. 4 : Southern blot of PCR product DNA from bovine urine samples. The blot was hybridized with genomic *L. hardjo* digoxygenin-11 dUTP labeled DNA probe. lane 1, urine sample seeded with .*hardjo* cells ; lane 2, urine sample with no leptospira added (negative control) .