A Comparison of Three DNA Extractive Procedures with *Leptospira* for Polymerase Chain Reaction Analysis

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Three DNA extraction methods were evaluated in this study: proteinase K followed by phenol-chloroform; a plant proteinase (E6870) followed by phenol-chloroform; and boiling of leptospires in 0.1 mM Tris, pH 7.0 for 10 min at 100°C, with no phenol treatment. Every strain treated with proteinase K or E6870 afforded positive polymerase chain reaction (PCR) reaction. On the other hand, from five strains extracted by the boiling method, three did not feature the 849 bp band characteristic in Leptospira. *We also evaluated by RAPD-PCR, DNAs from serovars isolated with proteinase K and proteinase 6870 with primers B11/B12. Each of the DNA samples provided PCR profiles in agreement with previous data. Moreover, the results with E6870 showed less background non-specific amplification, suggesting that removal of nucleases was more efficient with E6870. The limit for detection by PCR using Lep13/Lep14 was determined to be 10^2 leptospira, using the silver stain procedure.*

Key words: polymerase chain reaction - leptospira - DNA extraction

Leptospirosis is an important disease with worldwide distribution affecting wild and domestic animals as well as humans. In cattle, it causes economy losses with abortions, stillbirths, premature live birth and mastites. The main source of contamination is the shedding of *Leptospira* sp. through urine. The genus *Leptospira* belongs to the family Leptospiraceae, order Spirochateales (Johnson & Faine 1994). Conventional classification of leptospires relies on antigenic similarities with serovar as a basic taxon. So far, more than 200 serovars have been identified and grouped in 23 serogroups of antigenically related serovars. Domestic animals can be infected by any of the pathogenic serovars but each serovar usually remains in a specific maintenance host. Only a small number of serovars are endemic in any particular region or country (Ellis 1986). *Hardjo*, which is the serovar kept by cattle, has two genotypes, Hardjobovis and Hardjoprajitno, both found in cattle and sheep (Ellis 1994).

Current diagnosis methods for leptospirosis lack sensibility and specificity and are time consuming. Direct microscopic analysis of fresh urine has limitations for it needs relatively large number of leptospires for visualization. The serological method of choice is the microscopic agglutination test (MAT), but it fails to detect antibodies during chronic infections or in the early phase of the disease. Although culture techniques can be used to detect leptospires in urine, these procedures are slow, laborious and the samples are susceptible to contamination. For the diagnosis of leptospirosis, a rapid and sensitive assay must be able to detect leptospires in blood or urine of infected animals. The polymerase chain reaction (PCR) may fulfill these requirements. In recent years, PCR has been used to detect leptospires and other microorganisms in biological samples like urine (Van Eys et al. 1989, Gerritsen et al. 1991, Mérien et al. 1992, Bal et al. 1994, Brown et al. 1995), serum (Mérien et al. 1992, Gravekamp et al. 1993, Brown et al. 1995), liquor (Mérien et al. 1992, Romero et al.1998), milk (Zanini et al. 1998), and semen (Masri et al. 1997). The success of PCR depends on the quality of the DNA, that must be free of contaminants and nucleases that impair the amplification process. The addition of thyocianate, cetyltrimethyl ammonium bromide or phenol extraction to the specimen (Boom et al. 1990, Masri et al. 1997), are some of the options available. In a recent study we reported the application of a plant proteinase during the isolation of DNA (Genelhu et al. 1998). We compare here, three DNA extractive procedures in association with the PCR technique.

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MATERIALS AND METHODS

Strains of Leptospira sp. - Serovars of *Leptospira* sp. used in this work are listed in the Table. The strains were supplied by the Centro Panamericano of Zoonoses, Panamerican Health Organization. *L. interrogans* serovar hardjo reference strain Hardjoprajitno (CTG) and *L. borgpetersenii* serovar mini reference strain Cantagalo (CTG) were isolated at the Laboratory of Zoonoses, Departament of Preventive Medicine, Veterinary School of UFMG and classified at the Royal Tropical Institute (KIT), Amsterdan. The organisms were cultured in liquid Ellinghausen medium, modified by Johnson and Harris (1967), during seven days under aerobic conditions in the dark at 27°C.

DNA extraction - Exponentially growing organisms, were centrifuged at 13,000 x g for 30 min at 4°C, and the pellet was washed twice in phosphate-buffered saline (PBS). DNA was released from leptospires by either of the following methods: Method 1 - Incubation of bacteria in 200 µl of buffer containing 50 mM Tris (pH 8.0), 50 mM EDTA, 100 µM NaCl, 1% SDS and 100 mg of proteinase K (Sigma Chemicals, St. Louis, Missouri, USA) at 37°C for 2 h. DNA was then subjected to phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation. The precipitate was collected by centrifugation, then dried and resuspended in H_2O. In order to remove the contaminating RNA, the solution was incubated with 20 µg/ml of RNAase A (Amresco Inc., Solon, Ohio, USA) at 37°C for 2 h (Tamai et al. 1988); Method 2 - Incubation of leptospires in the buffer above described plus 50 µg of a plant proteinase (E6870) (Genelhu et al. 1998) during 2 h at 37°C, followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) as described for Method 1; Method 3 - Boiling of 1 ml of leptospire culture in 100 µl of 0.1 mM Tris (pH 7.0) for 10 min at 100°C (Corney et al. 1993).

The amount of DNA recovered after each procedure was determined by electrophoresis on a 1% agarose gel against λ *Hin*d III DNA. The gel was stained with ethidium bromide and the DNA concentration was determined by direct comparison with λDNA as standard.

PCR - The PCR was undertaken with the primers previously described by Woodward and Redstone (1993): Lep 13, 5'CTCGGATCCTTAG ATATGCTGCAGAAGCTTG 3'and Lep 14, 5'AA AAGATCTTATGATTATGATCACAACCTG 3'. The reaction mix (25 µl) contained 10 mM Tris-HCl (pH 9.0) buffer, 50 mM KCl, 100 µM of each deoxynucleoside triphosphate, 800 nM of each primer, 0.2 ng of DNA, 2 mM $MgCl_2$ and 0.5 U of *Taq* DNA polymerase (Gibco). The mix was covered with 15 µl of mineral oil and PCR was performed in a Thermocycle MJ Research for 30 sequential cycles of 94°C for 90 s, 58°C for 90 s and 72°C 2 min. The last elongation step was extended for 10 min. With this protocol, the presence of a 849 bp band indicates the successful amplification of the endoflagellin subunit gene (Woodward & Redstone 1993) acting as a target sequence for the detection of the genus *Leptospira*.

Also, the DNA released with proteinase K and proteinase E6870 were used in association with RAPD-PCR protocols, to check for the ability to differentiate between serovars of *Leptospira* and to verify the quality of DNA obtained with E6870. We used a different set of primers, previously described by (Ramadass et al. 1997), B11; 5'CCGGAAGAAGGGGCGCCAT3' and B12; 5'CGATTTAGAAGGACTTGCACAC 3'. The RAPD-PCR reaction (25 µl) was performed in 10 mM Tris-HCl (pH 9.0) buffer, containing 50 mM KCl, 100 µM each deoxynucleoside triphosphate, 4 nM each primer, 50 ng of DNA, 6 mM $MgCl_2$ and 0.5 U of *Taq* DNA Polimerase (Gibco). The reaction mixtures were covered with 15 µl of mineral oil and PCR was performed in a Thermocycle MJ Research. The first two cycles consisted of denaturation at 95°C for 5 min, annealing of primers at 40°C for 5 min, and extension at 72°C for 5 min. The subsequent 35 cycles consisted of denaturation at 95°C for 1 min, annealing of primers at 58°C for 1 min and extension at 72°C for 3 min.

TABLE

Leptospira sp. serovars used in the study

Genomic species	Serogroup	Serovar	Reference sample
L. interrogans	Australis	bratislava	Jez bratislava
L. interrogans	Pomona	pomona	Pomona
L. interrogans	Sejroe	hardjo	Hardjoprajitno (OMS)
L. interrogans	Sejroe	hardjo	Hardjoprajitno (CTG)
L. borgpetersenii	Mini	mini	Cantagalo (CTG)
L. borgpetersenii	Mini	szwajizak	Szwajizak
L. borgpetersenii	Tarassovi	tarassovi	Perepelicin

The last elongation step was extended for 10 min.

Following PCR the amplified DNA were deproteinized by phenol extraction and ethanol precipitation before further analysis.

Electrophoresis of PCR products - The amplified fragments were resolved on a 6% PAGE gel for 45 min at 100 V. Following electrophoresis the DNA was visualized by silver staining (Sanguinetti et al. 1994).

Serial dilution of Leptospira into urine - To determine the sensitivity of PCR with primers Lep13/Lep14, grown-up cultures of *Leptospira* were quantified using a Neubauer chamber, followed by 10-fold serial dilution (10^9 to 10^1) of *L. hardjo*/Hardjoprajitno (CTG) into 1 ml aliquots of urine.

RESULTS AND DISCUSSION

A comparison of three extracting procedures was carried out to determine which method yields more reliable results to be adopted in the future as standard procedure for diagnosis of *Leptospira*. PCR with primers Lep13/Lep14 was done after DNA was released by either, of the three methods outlined in the Methods section. Using proteinase K or E6870, it was observed a main band of 849 bp in each of the serovars assayed, in agreement with previous data (Woodward & Redstone 1993) (Fig. 1A-C). In addition, a larger band (>2kb) was observed in some serovars (*szwayizak, mini, pomona* and *hardjo/Hardoprajitno*) regardless of the extracting procedure. Also, a 300 bp was seen in serovars hardjo/Hardoprajitno CTG and OMS extracted with plant proteinase. With the exception of *L. borgpetersenii* serovar szwayizak the intensisty of the 849 bp specific for *Leptospira* obtained with plant proteinase is stronger than that obtained with proteinase K. The densitometric analysis of the electrophoretogram (not shown) confirms this observation (Fig. 1A, B). The variation in the band intensities observed in some isolates, could be explained by differential amounts of DNA substrate added to the PCR reaction, although each DNA sample following extraction was dosed by agarose gel electrophoresis before PCR. Alternatively, these differences can be attributed to remaining contaminants within the DNA preparation that inhibit the PCR reaction (Longo et al. 1990). The improved DNA protection by E6870 can be explained by the presence of EDTA in the extracting buffer. The chelating agent, favors the proteolytic activity of E6870, while hampering the activity of proteinase K. This notion was confirmed when the intactness of DNA obtained with plant proteinase was compared by agarose electrophoresis with the DNA extracted with proteinase K, the former showing less sign of degradation than the latter.

On the other hand, no PCR products were obtained in some of the serovars samples [szwajizak, hardjo (OMS) and pomona] extracted by the boiling procedure (Fig. 1C). To verify whether the negative PCR resulting from this procedure is due to inefficient thermal lysis, the extracts subjected to this procedure were deproteinized by phenol-chloroform and the nucleic acids concentrated by ethanol precipitation. The identity and relative size of the DNA recovered was evaluated on 1% agarose gel electrophoresis using l DNA *Hin*d III as molecular weight marker (not shown). While the

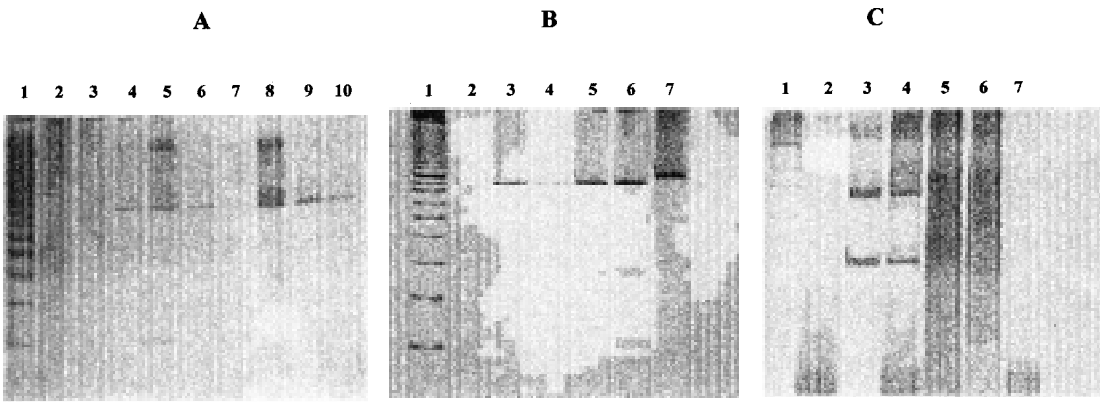

Fig. 1A-C: polyacrylamide gel electrophoresis of *Leptospira* DNA amplified by polymerase chain reaction (PCR) - Fifty percent (12.5 µl) of the PCR reaction from various serovars amplified with primers Lep13/Lep14 as described in the Methods section were resolved by 6% non denaturing PAGE. A: serovars extracted with proteinase K; B: serovars extracted with E6870; C: serovars extracted by 10 min boiling of samples. A: lane 1, 100 bp; lane 2 and 3, negative control; lanes 4-10 are szwajizak, mini, hardjo (OMS), *hardjo* (CTG), *pomona*, *bratislava*, *tarassovi;* B: lane 1, 100 bp; lane 2, negative control; lane 3, positive control; lanes 4-7 are *szwajizak*, *mini*, *hardjo* (OMS), *hardjo* (CTG); C: lane 1, 100 bp; lane 2, negative control; lanes 3-7, *mini*, *bratislava*, *hardjo/ Hardjoprajitno* (OMS); *szwajizak*, *pomona*, respectively.

PCR-positive samples obtained by the boiling procedure appear as faint smear, ranging in size from 2.3kb-100bp, a lack of DNA was evident in samples affording a negative PCR. We conclude from these results and from previous data, that while *Leptospira* serovars *sejroe* and *hardjo,* genotype Hardjobovis (Van Eys et al. 1989), serovars *pomona, balcanica and hardjo* (genotypes Hardjoprajitno and Hardjobovis) (Corney et al. 1993) release their DNA, other serovars are inefficiently disrupted using the boiling method. These experiments were repeated several times with different culture batches, yet the results showed the same profile described earlier; i.e. the PCR positive serovars continued to yield positive results, while refractory serovars showed no PCR amplification. These results suggest that differences in the cell wall structure may account for the variation in sensitivity to the boiling procedure. A similar situation has been previously reported in Mohran et al. (1998) with *Campylobacter* isolates.

In addition, the proteinase K and the plant proteinase procedures were used in combination with a different protocol, the RAPD-PCR of DNA from *Leptospira.* Using proteinase K in five strains or the plant proteinase in four strains of *Leptospira* with primers B11/B12, resulted in amplification of several bands ranging in size between 100 to 1000 bp (Fig. 2A, B). Again, the profiles obtained with serovar hardjo/ Hardjoprajitno match the results previously reported. Also, the profiles obtained with E6870 (Fig. 3), showed less background than those obtained with proteinase K, (Fig. 2A, B), suggesting that DNA extracted with plant proteinase was more intact. Two of the strains in these experiments have not been previously characterized by the RAPD-PCR technique [*L. borgpetersenii* serovar mini and *L. interrogans* serovar hardjo/Hardjoprajitno (CTG)].

Finally, the sensitivity of the PCR protocol involving DNA extraction with plant proteinases was evaluated by serially diluting (10^{10} to 10^{1}) live leptospira into urine from cattle, followed by DNA extraction and PCR as described in the Methods section. The results of these experiments shown in Fig. 3 reveal that up to 100 *Leptospira* could be

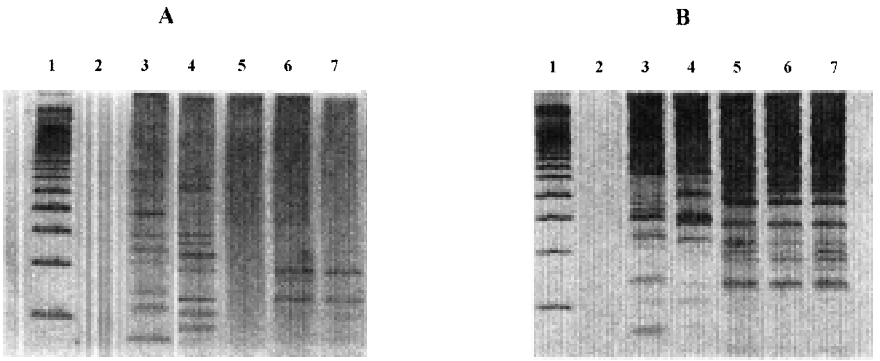

Fig. 2A-B: polyacrylamide gel electrophoresis of *Leptospira* DNA amplified by RAPD-polymerase chain reaction (PCR) - Fifty percent (12.5 µl) of the PCR reaction from various serovars amplified with primers B11/B12 as described in the Methods section were resolved by 6% non denaturing PAGE. A: serovar extraction with proteinase K; B: serovar extraction with E6870; A: lane 1, 100 bp; lane 2, negative control; lanes 3-7 are *szwajizak*, *mini*, *hardjo* (OMS), *hardjo* (CTG), *bratislava.*

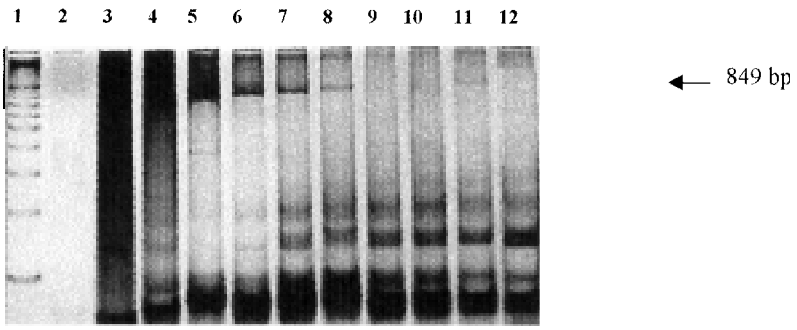

Fig. 3: polyacrylamide gel electrophoresis of serially diluted *Leptospira* in cattle urine followed by polymerase chain reaction (PCR) amplification - Fifty percent (12.5 µl) of the PCR reaction from serovar *hardjo*/*Hardjoprajitno* (CTG) amplified with primers Lep13/Lep14 as described in the Methods section were resolved by 6% non denaturing PAGE. lane 1, 100 bp; lane 2, negative control; lanes 3-12 are 10^{10}, 10^{9}, 10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4}, 10^{3}, 10^{2}, 10^{1} cells/ml, respectively.

detected by using this procedure. This result supports the potential of the PCR procedure when applied to the diagnosis of *Leptospira*, as infected cattle secret about 10^5 microorganisms/ml urine during the infected state.

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