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DIFFERENTIATION OF PATHOGENIC AND NON-PATHOGENIC LEPTOSPIRES BY MEANS OF THE POLYMERASE CHAIN REACTION

Alberto E. PARMA(1), Alfredo SEIJO(2), Paula M. LUCCHESI(1), Bettina DEODATO(2) & Marcelo E. SANZ(1)

SUMMARY

A polymerase chain reaction was carried out to detect pathogenic leptospires isolated from animals and humans in Argentina. A double set of primers (G1/G2, B64-I/B64-II), described before, were used to amplify by PCR a DNA fragment from serogroups belonging to *Leptospira interrogans* but did not allow to detect saprophytic strains isolated from soil and water (*L. biflexa*). This fact represents an advantage since it makes possible the differentiation of pathogenic from non-pathogenic leptospires in cultures. The sensitivity of this assay has been determined, allowing to detect just only 10 leptospires in the reaction tube. Those sets of primers generated either a 285 bp or 360 bp fragment, depending on the pathogenic strain.

KEYWORDS: Polymerase chain reaction; *Leptospira biflexa*; *L. interrogans.*

INTRODUCTION

Leptospirosis is a widespread disease that affects wild and domestic animals as well as humans[1]. There are several reports on the frequency of leptospirosis among livestock, wild animals and human beings in South America mainly in Argentina[2] and Brazil[15,18]. This makes this study very important for our continent. Some infected animals develop persistent renal infections and shed leptospires in their urine[23]. Systematic taxonomy of the leptospires distributes these bacteria between two species, *Leptospira interrogans* (pathogenic) and *L. biflexa* (saprophytic) comprising over 200 serovars[7] on the basis of surface agglutinins. The taxonomy of the pathogenic leptospires has been recently reorganized, on the basis of studies on DNA homology, polymorphic patterns and rRNA typing, into seven genospecies: *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai*, *L. inadai* and *L. kirschneri*[13,14,29].

The presence of the bacterium can be investigated by culture, however, this procedure might require up to 2 months[4]. The detection of specific antibodies is worldwide performed by microscopic agglutination test and, complementary, by enzyme-linked immunosorbent assay[12,25]. Looking for a faster detection, several methods have been carried out such as immuno-fluorescence[20,26], immunoperoxidase staining[21] and DNA hybridization with oligonucleotide probes[10,22]. In spite of their usefulness for the detection of leptospires, these techniques require the microorganism to be present in relatively large numbers. Nowadays, with the introduction of the polymerase chain reaction (PCR)[16], a rapid detection of small numbers of leptospires in clinical samples may became practical due to specific amplification of leptospiral DNA. In fact, oligo-nucleotide primers have been developed by several research teams for the specific detection of certain serovars. PCR was applied to identify the serovar *hardjo*, genotype *bovis*[24,28]. When combined with restriction enzyme analysis, PCR allowed to distinguish among serovars[19]. By using oligonucleotide primers designed from the 16S rRNA gene of serovar *canicola*, it was possible to detect leptospires although without differentiating between pathogenic and saprophytic species[9]. Later, some oligonucleotide primers were developed from genomic libraries constructed with DNA from *icterohaemorrhagiae* and *bim* serovars,allowing to detect only pathogenic leptospires[6].

The casual presence of *L. biflexa* in the primary cultures performed from clinical samples, in addition to the low growing rate of *L. interrogans in vitro*, makes the isolation and characterization of pathogenic leptospires difficult and

(1) Laboratorio de Inmunoquímica y Biotecnología, FCV, UNCPBA, Tandil, Argentina.
(2) Servicio de Zoonosis, Hosp. de Infecciosas "Dr F.J. Muñiz", Buenos Aires, Argentina.
Correspondence to: Dr Alberto E. Parma, Lab. Inmunoquímica y Biotecnología, FCV. Univ. Nac. del Centro Prov. Bs. As., Pinto 399 (7000) Tandil, Argentina. e-mail: aparma@vet.unicen.edu.ar

troublesome. This indicates the interest for an alternative method to differentiate pathogenic from saprophytic leptospires which has to be rapid, specific and sensitive. By using different oligonucleotide primers described in the literature, in this work we tried to develop a polymerase chain reaction to be applied to the analysis of strains isolated in Argentina.

MATERIALS AND METHODS

Organisms and growth conditions

Twenty nine strains belonging to *L. biflexa*, most of which had been isolated from water and soil of Buenos Aires and Jujuy (Argentina), and 31 pathogenic strains (reference strains and clinical isolates from this country) were grown at 30°C in polysorbate 80-bovine albumin medium (EMJH)[3] and maintained in Fletcher medium[5]. Stock laboratory cultures were maintained by regular subculture into fresh medium. Strains were typed by the cross-agglutination absorption test[27] and biochemical assays.

Sensitivity assay

Aliquots were taken from a *L. interrogans* serovar *pomona* culture in EMJH. Cells were washed twice in sterile distilled water by centrifugation at 4° C – 10,000 rpm (microcentrifuge Sorvall RMC 14). After estimating the bacterial concentration by nephelometry, the suspension was brought to 1, 10, 100, 1000 and 10000 bacteria per 5 µl and Tween 20 was added to a final concentration of 1%. The suspensions were boiled for 10 min. Five µl from each one were assigned to DNA amplification.

With the aim to establish the limit of PCR sensitivity expressed in µg of DNA, this molecule was purified from *L. interrogans* serovar *pomona* by means of phenol-chloroform extraction and precipitated with 300 mM sodium acetate and 70% ethanol. Lastly, DNA was washed with 70% ethanol, redissolved in sterile distilled water and its concentration was adjusted by reading the absorbance at 260 nm[17].

Sample preparation from isolates

The reaction mix was constituted by 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% (v/v) Triton X-100, 2.5 mM $MgCl_2$ 0.01% (m/v) gelatine, 250 µM each dNTP, 0.5 µM each primer (G1, G2, B64-I, B64-II)[6], 1 U *Taq* DNA polymerase (Promega). Each tube received 5-10 µl of the DNA solution obtained as indicated above. Finally, one drop of mineral oil was added to each tube to prevent evaporation. The thermocycler was programmed as follows: one initial cycle of 94° C for 180", one final cycle of 72° C for 240" and 38 cycles of 60" at 94° C (denaturing), 60" at 57° C (annealing) and 150" at 72° C (extension). Another annealing temperature was assayed (55° C) keeping the remaining conditions steady. T_m (melting temperature) for each primer was calculated and analysed with the computer software Oligo 4.0 (Primer Analysis Software, National BioSciences).

Agarose gel electrophoresis

Ten µl aliquots from each DNA amplification were analysed by horizontal agarose gel electrophoresis and UV transillumination (300 nm). Gel was constituted by 1.5% (m/v) agarose and 1.2 µg ml^{-1} ethidium bromide in running buffer (89 mM Tris, 89 mM boric acid, 1 mM EDTA, pH 8.0). DNA Ladder (100 bp – Promega) was used as molecular size marker for the range 100-1500 bp.

RESULTS

The melting temperature (T_m) for each oligonucleotide primer was calculated and analysed with the computer software Oligo 4.0 using both, the simple formula $T_m = 2(A+T) + 4(G+C)$ and that based on % (G+C). The melting temperatures obtained using the first manual method showed a dispersion of values among primers of 4.0°C (54 to 58°C), meanwhile in those calculated taking into account the % (G+C) content the dispersion was 4.1°C (62.1 to 66.2°C). By consensus between both methods it was possible to obtain an annealing temperature of 57°C. When the annealing was performed at 55°C it was obtained a weak background in the amplification of some *L. biflexa* strains. Temperature profiles for DNA amplification were experimentally determined to obtain a satisfactory sensitivity.

Several amounts (5000, 500, 50, 5 and 0.5 pg) of purified DNA obtained from *L. interrogans* serovar *pomona* were used as a target to be amplified by PCR. Although with some difficulty, it was possible to perceive an amplimer corresponding to 285 bp by agarose gel electrophoresis when only 0.5 pg of leptospiral DNA was used (Fig. 1).

Sensitivity was also evaluated taking into account the minimal number of microorganisms that had to be added to the PCR mix to produce a noticeable signal. It was found that only 10 microorganisms (*L. interrogans* serovar *pomona*) could be detected by this reaction system using the primers G1/G2 (not shown). These oligonucleotide primers allowed to amplify a

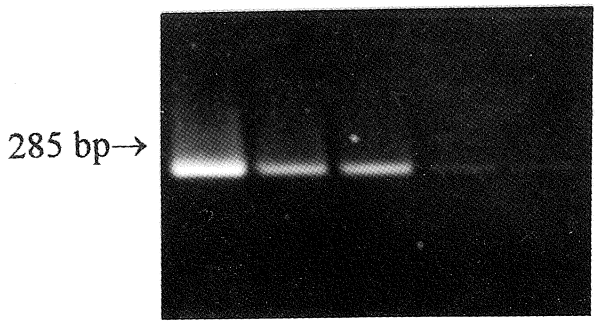

Fig. 1 – Sensitivity of the amplification of *L. interrogans* serovar *pomona* DNA by PCR. From left to right 5000, 500, 50, 5 and 0.5 *pg* of purified DNA. Amplimer sizes correspond to 285 bp. Gel: 1.5% (m/v) agarose.

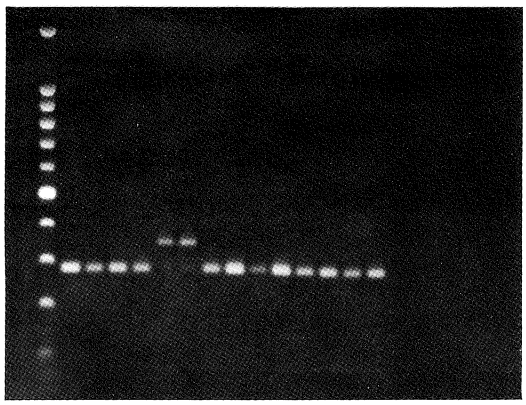

Fig. 2 – Agarose gel showing the amplification by PCR of DNA from several pathogenic and saprophytic leptospires. Line 1: molecular size marker, DNA Ladder (100 bp – Promega) for the range 100-1500 bp; 2: *bataviae* (Van Tienen); 3. *canicola* (Hond Utrecht IV); 4: canicola (isolation Y); 5. *sentot* (Sentot); 6: Grippotyphosa (isolation 782 Muñiz); 7: Grippotyphosa (isolation G); 8: *hebdomadis* (Hebdomadis); 9: *icterohaemorrhagiae* (RGA); 10: *icterohaemorrhagiae* (isolation Rata Y); 11: *pomona* (Pomona); 12: *pomona* (Corrientes); 13: *pyrogenes* (Salinem); 14: *hardjo* (Hardjoprajitno); 15: *wolffi* (3705); 16: *L. biflexa* (Patoc I); 17-20: *L. biflexa* (isolations Pergamino, 2243 Muñiz, 2360 Muñiz, Patoc patoc).

285 bp fragment from all the pathogenic *Leptospira* strains assayed (Fig. 2), except those strains corresponding to Cynopteri and Grippotyphosa serogroups. On the other hand, the oligonucleotide primers B64-I and B64-II enabled the amplification of strains belonging to these serogroups, producing an amplimer of 360 bp. When used together, these four primers allowed to amplify DNA from all pathogenic leptospires mentioned above (Table 1). On the contrary, DNA samples from all the 29 saprophytic strains belonging to *L. biflexa* could not be amplified by means of these four primers. These leptospires had been classified by means of biochemical assays (8-azaguanine-supplemented medium).

DISCUSSION

It is known that increasing the annealing temperature, without exceeding the melting temperature, enhances discrimination against incorrectly annealed primers and reduces misextension of incorrect nucleotides at the 3'end of primers[8]. Therefore, stringent annealing temperatures, especially during several of the first cycles, would help to increase specificity. The annealing temperature compatible with the four primers was 50°C when the method for calculation of T_m was that based on the formula 2(A+T) + 4(G+C). Nevertheless, when considered the formula based on %(G+C), the annealing temperature might arise to 57°C. A lightly lower value (55°C) was assayed as well with the aim to avoid too stringent conditions for the primers with the lower T_m. However, the amplification performed by annealing the primers at 57°C rendered the cleanest

TABLE 1
Leptospira strains studied by PCR. Shadow indicates strains amplified by PCR. ND: not determined.

Serogroup	Serovar	Strain	Serogroup	Serovar	Strain
L. interrogans			*L. biflexa*		
Autumnalis	autumnalis	Akiyami A	ND	ND	isolat. 1-Muñiz
Ballum	castellonis	Castellon 3	ND	ND	isolat. 2-Muñiz
Bataviae	bataviae	Van Tienen	ND	ND	isolat. 3-Muñiz
Canicola	canicola	Hond Utrecht IV	ND	ND	isolat. 4-Muñiz
Cynopteri	cynopteri	3522C	ND	ND	isolat. 5-Muñiz
Djasiman	sentot	Sentot	ND	ND	isolat. 6-Muñiz
Hebdomadis	hebdomadis	Hebdomadis	ND	ND	isolat. 7-Muñiz
Icterohaemorrhagiae	icterohaemorrhagiae	RGA	ND	ND	isolat. 8-Muñiz
Javanica	javanica	Veldrat Batavia 46	ND	ND	isolat. 9-Muñiz
Panama	panama	CZ 214K	ND	ND	isolat. 10-Muñiz
Pomona	pomona	Pomona	ND	ND	isolat. 11-Muñiz
Pyrogenes	pyrogenes	Salinem	ND	ND	isolat. 12-Muñiz
Sejroe	hardjo	Hardjoprajitno	ND	ND	isolat. 13-Muñiz
Sejroe	wolffi	3705	ND	ND	isolat. 14-Muñiz
Tarassovi	tarassovi	Perepelicin	ND	ND	isolat. 15-Muñiz
Pomona	ND	Corrientes	ND	ND	isolat. 16-Muñiz
Pomona	ND	Bayur	ND	ND	isolat. 17-Muñiz
Pomona	ND	isolation A	ND	ND	isolat. 18-Muñiz
Pomona	ND	isolation B	ND	ND	isolat. 19-Muñiz
Pomona	ND	isolation C	ND	ND	isolat. 20-Muñiz
Pomona	ND	isolation D	ND	ND	isolat. 21-Muñiz
Canicola	ND	C85K4	ND	ND	isolat. 22-Muñiz
Canicola	ND	isolation I	ND	ND	isolat. 23-Muñiz
Canicola	ND	isolation J	ND	ND	isolat. 24-Muñiz
Grippotyphosa	ND	782 – Muñiz	Semaranga	patoc	Patoc I
Grippotyphosa	ND	isolation G	ND	ND	isol. 2360-Muñiz
Grippotyphosa	ND	isolation H	ND	ND	isol. Pergamino
Icterohaemorrhagiae	ND	isolation Rata I	ND	ND	isol. 2243-Muñiz
Icterohaemorrhagiae	ND	isolation E	ND	ND	isol. Patoc patoc
Icterohaemorrhagiae	ND	isolation F			

electrophoretic pattern. Obviously, this change made the assay more specific allowing the primers to hybridize only to the sequences with the highest homology. The amplimer we obtained from some pathogenic *Leptospira* strains by using G1/G2 primer pair corresponded to the expected 285 bp fragment, whereas B64-I/B64-II generated a 360 bp fragment from the remaining strains tested instead of the 563 bp fragment obtained by GRAVEKAMP et al.[6]. This fact represents an interesting finding which deserves further studies. The size of the 360 bp fragment was not modified whether the B64-I/B64-II primers were used alone or together with the other pair of primers.

DNA obtained from other microorganisms such as *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Mycobacterium* spp., *Salmonella* sp. group B, *Yersinia enterocolitica*, *Escherichia coli*, *Borrelia burgdorferi* and *Treponema reiteri*, could not be amplified under these conditions[6]. The specificity that this reaction showed in the presence of other bacterial species indicates it is suitable for the analysis of primary cultures of leptospires.

Far from being considered as an inconvenience, the inability of this set of primers to detect saprophytic leptospires, belonging to *L. biflexa*, has to be considered as an important operative advantage. In fact, one of the main time consuming tasks in the leptospirosis laboratory is to determine both species and serogroups to which an isolated leptospire corresponds to and its differentiation from those previously known as non-pathogenic ones. This reaction allows a rapid and sensitive differentiation of pathogenic from saprophytic leptospires in culture.

PCR may be inhibited by a wide list of substances such as hemoglobin, heparin, sodium ion, phosphate, EDTA, citrate, high concentration of magnesium[8]. Consequently, the application of this assay to the analysis of clinical samples still requires further studies including the overcoming of the action of inhibitors and the confrontation with current laboratory methods.

PCR has already been shown to be capable of detecting leptospires in serum and urine of infected patients[6,9,24] and appears to be a promising method for early diagnosis[11].

In spite of the advances made many variables have to be controlled before this assay becomes a routine diagnostic procedure.

RESUMO

Diferenciação das leptospiras patogênicas e não patogênicas por PCR

Utilizou-se a reação em cadeia da polimerase (PCR) para identificar leptospiras patogênicas isoladas, na Argentina, de animais e do homem. Foram usados dois pares de primers (G1/G2; B64-I/B64-II), descritos anteriormente como apropriados para amplificar amostras pertencentes aos diferentes sorogrupos de *Leptospira interrogans*. Através deste método não se detectaram as leptospiras saprófitas (*L. biflexa*) isolados de água e solo. Este fato representa uma vantagem uma vez que possibilita a diferenciação de leptospiras patogênicas das não patogênicas em culturas. A sensibilidade da prova foi determinada, verificando-se que ela permitiu detectar 10 leptospiras por tubo de reação. Os tamanhos dos fragmentos amplificados foram de 285 ou 360 pares de bases (bp), dependendo da amostra patogênica estudada.

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