Detection of seven species of pathogenic leptospires by PCR using two sets of primers

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Two sets of primers derived from genomic DNA libraries of *Leptospira* serovars *icterohaemorrhagiae* (strain RGA) and *bim* (strain 1051) enabled the amplification by PCR of target DNA fragments from leptospiral reference strains belonging to all presently described pathogenic *Leptospira* species. The *icterohaemorrhagiae*-derived primers (G1/G2) enabled amplification of DNA from *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai* and *L. meyeri*, whereas the *bim*-derived primers (B64-I/B64-II) enabled the amplification of *L. kirschneri*. Southern blot and DNA sequence analysis revealed inter-species DNA polymorphism within the region spanned by primers G1 and G2 between *L. interrogans* and various other *Leptospira* species. Using a mixture of primer sets G1/G2 and B64-I/B64-II, leptospires of serovars *icterohaemorrhagiae*, *copenhageni*, *hardjo*, *pomona*, *grippityphosa* and *bim* were detected in serum samples collected from patients during the first 10 days after the onset of illness.

Introduction

Leptospirosis is probably the world's most widespread zoonosis (Turner, 1970). Early treatment with antibiotics and haemodialysis have contributed to the decrease of mortality (Centers for Disease Control, 1981; Sanford, 1984). Rapid diagnosis of leptospirosis is important in view of an adequate early treatment. Clinically, it is of sufficient importance to be able to state if a patient is infected with pathogenic leptospires or not. Epidemiologically, it is of added value if one can state the nature of the causative leptospiral strain. Conventional classification at the subspecies level of leptospires is based on antigenic similarities with the serovar as a basic taxon. Thus far, more than 200 serovars have been identified divided over 23 serogroups of antigenically similar serovars (Kmety & Dikken, 1988).

In 1987, Yasuda *et al.* proposed a new classification of the genus *Leptospira* in which the old pathogenic species

L. interrogans and the saprophytic *L. biflexa* each were subdivided into a number of new species. This genetic classification, which is based on DNA homology and does not follow distinctions or clustering based on antigenic similarities, divides leptospiral strains into four non-pathogenic species, *L. biflexa*, *L. meyeri*, *L. parva*, and *L. wolbachii*, and seven pathogenic species, *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai*, *L. inadai*, and *L. kirschneri* (Ramadass *et al.*, 1992). *L. kirschneri* corresponds largely to an unofficially named species *L. alstoni* (A. F. Kaufmann, personal communication).

Clearly, serology does not contribute to early diagnosis of leptospirosis as antibodies become detectable on approximately the seventh day of disease. Leptospires circulate through the blood of a patient until approximately the 10th day after the onset of symptoms. Conventional methods to detect leptospires in blood such as dark field microscopy or culturing are either unreliable or too slow to contribute to an early diagnosis (Turner, 1970; Faine, 1982). Immunofluorescence staining (Sheldon, 1953; White & Ristic, 1959), immunoperoxidase staining (Terpstra *et al.*, 1983) and DNA hybridization (Terpstra *et al.*, 1986) are useful methods

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Abbreviations: GuSCN, guanidinium thiocyanate; MAT, microscopic agglutination test.

Table 1. *Leptospiral strains tested by PCR*

All strains were tested two or more times and gave reproducible results.

Serogroup	Serovar	Strain*	Serogroup	Serovar	Strain*
<i>Leptospira interrogans</i> †			<i>Leptospira borgpetersenii</i> cont.		
Australis	<i>australis</i>	Ballico	Mini	<i>mini</i>	Sari
Australis	<i>bangkok</i>	BD 92	Pyrogenes	<i>kwale</i>	Julu
Australis	<i>bratislava</i>	Jez Bratislava	Sejroe	<i>balcanica</i>	1627 Burgas
Australis	<i>fugis</i>	Fudge	Sejroe	<i>istrice</i>	Bratislava
Australis	<i>hawaii</i>	LT 62-68	Sejroe	<i>nyanza</i>	Kibos
Australis	<i>jalna</i>	Jalna	Sejroe	<i>polonica</i>	493 Poland
Australis	<i>lora</i>	Lora	Sejroe	<i>sejroe</i>	M 84
Autumnalis	<i>autumnalis</i>	Akiyami A	Tarassovi	<i>guidae</i>	RP 29
Autumnalis	<i>bangkinang</i>	Bangkinang I	Tarassovi	<i>kisuba</i>	Kisuba
Autumnalis	<i>mooris</i>	Moore	Tarassovi	<i>tarassovi</i>	Perepelicin
Autumnalis	<i>rachmati</i>	Rachmat	Tarassovi	<i>tunis</i>	P 2/65
Autumnalis	<i>weerasinghe</i>	Weerasinghe	<i>Leptospira weilii</i> †		
Bataviae	<i>bataviae</i>	Swart	Celledoni	<i>celledoni</i>	Celledoni
Bataviae	<i>paidjan</i>	Paidjan	Celledoni	<i>hainan</i>	6712
Canicola	<i>benjamini</i>	Benjamin	Javanica	<i>mengrun</i>	A 102
Canicola	<i>bindjei</i>	Bindjei	Javanica	<i>coxi</i>	Cox
Canicola	<i>broomi</i>	Patane	Manhao	<i>qingshui</i>	L 105
Canicola	<i>canicola</i>	Hond Utrecht IV	Sarmin	<i>sarmin</i>	Sarmin
Canicola	<i>kuwait</i>	136/2/2	Tarassovi	<i>langati</i>	M 39090
Canicola	<i>portlandvere</i>	MY 1039	Tarassovi	<i>mogdeni</i>	Compton 746
Canicola	<i>schueffneri</i>	Vleermuis 90 C	<i>Leptospira noguchii</i> †		
Djasiman	<i>djasiman</i>	Djasiman	Australis	<i>peruviana</i>	LT 941
Djasiman	<i>gurungi</i>	Gurung	Autumnalis	<i>fortbragg</i>	Fort Bragg
Djasiman	<i>sentot</i>	Sentot	Bataviae	<i>argentiniensis</i>	Peludo
Grippotyphosa	<i>valbuzzi</i>	Valbuzzi	Bataviae	<i>claytoni</i>	1348 U
Hebdomadis	<i>hebdomadis</i>	Hebdomadis	Louisiana	<i>louisiana</i>	LSU 1945
Hebdomadis	<i>kremastos</i>	Kremastos	Louisiana	<i>orleans</i>	LSU 2580
Icterohaemorrhagiae	<i>birkini</i>	Birkin	Mini	<i>beye</i>	1537 U
Icterohaemorrhagiae	<i>copenhageni</i>	M 20	Panama	<i>cristobali</i>	1996 K
Icterohaemorrhagiae	<i>gem</i>	Simon	Panama	<i>panama</i>	CZ 214 K
Icterohaemorrhagiae	<i>icterohaemorrhagiae</i>	RGA	Pomona	<i>proechimys</i>	1161 U
Icterohaemorrhagiae	<i>lai</i>	Lai	Shermani	<i>carimagua</i>	9160
Icterohaemorrhagiae	<i>mankarso</i>	Mankarso	<i>Leptospira santarosai</i> †		
Icterohaemorrhagiae	<i>naam</i>	Naam	Ballum	<i>peru</i>	MW 10
Icterohaemorrhagiae	<i>smithi</i>	Smith	Bataviae	<i>balboa</i>	735 U
Louisiana	<i>lanka</i>	R 740	Bataviae	<i>brasilensis</i>	An 776
Mini	<i>szwajizak</i>	Szwajizak	Bataviae	<i>kobbe</i>	CZ 320
Pomona	<i>pomona</i>	Pomona	Cynopteri	<i>tingomaria</i>	M 13
Pyrogenes	<i>abramis</i>	Abraham	Grippotyphosa	<i>bananal</i>	Aa14
Pyrogenes	<i>biggis</i>	Biggs	Grippotyphosa	<i>canalzonae</i>	CZ 188
Pyrogenes	<i>camlo</i>	LT 64-67	Hebdomadis	<i>borincana</i>	HS 622
Pyrogenes	<i>manilae</i>	LT 398	Hebdomadis	<i>maru</i>	CZ 285
Pyrogenes	<i>pyrogenes</i>	Salinem	Hebdomadis	<i>sanmartini</i>	CT 63
Pyrogenes	<i>robinsoni</i>	Robinson	Javanica	<i>fluminense</i>	Aa 3
Pyrogenes	<i>zanoni</i>	Zanoni	Javanica	<i>vargonicas</i>	24
Ranaru	<i>evansi</i>	267-1348	Mini	<i>georgia</i>	LT 117
Sejroe	<i>geyaweera</i>	Geyaweera	Mini	<i>ruparupae</i>	M 3
Sejroe	<i>haemolytica</i>	Marsh	Mini	<i>tabaquite</i>	TRVL 3214
Sejroe	<i>hardjo</i>	Hardjoprajitno	Pomona	<i>tropica</i>	CZ 299
Sejroe	<i>medanensis</i>	Hond HC	Pryogenes	<i>alexi</i>	HS 616
Sejroe	<i>ricardi</i>	Richardson	Pryogenes	<i>princestown</i>	TRVL 112499
Sejroe	<i>romantica</i>	LM 294	Pryogenes	<i>varela</i>	1019
Sejroe	<i>wolfii</i>	3705	Sarmin	<i>machiguenga</i>	MMD 3
<i>Leptospira borgpetersenii</i> †			Sarmin	<i>rio</i>	Rr 5
Autumnalis	<i>srebarna</i>	1409/69	Sarmin	<i>weaveri</i>	CZ 390
Ballum	<i>ballum</i>	Mus 127	Sejroe	<i>caribe</i>	TRVL 61866
Ballum	<i>guangdong</i>	1853	Sejroe	<i>gorgas</i>	1413 U
Ballum	<i>castellonis</i>	Castellon 3	Sejroe	<i>guaricura</i>	Bov.G
Celledoni	<i>whitcombi</i>	Whitcomb	Sejroe	<i>trinidad</i>	TRVL 34056
Javanica	<i>ceylonica</i>	Piyasena	Shermani	<i>babudieri</i>	CI 40
Javanica	<i>javanica</i>	Veldrat Bat 46	Shermani	<i>hais</i>	M 6
Javanica	<i>sorexjalna</i>	Sorex Jalna	Shermani	<i>shermani</i>	1342 K
Javanica	<i>yaan</i>	80-27			

Table 1. (cont.)

Serogroup	Serovar	Strain*	Serogroup	Serovar	Strain*
<i>Leptospira santarosai</i> cont.			<i>Leptospira kirschneri</i> cont.		
Tarassovi	<i>atlantae</i>	LT 81	Grippytyphosa	<i>ratnapura</i>	Wumalasena
Tarassovi	<i>bakeri</i>	LT 79	Grippytyphosa	<i>vanderhoeveni</i>	Kipod 179
Tarassovi	<i>chagres</i>	1913 K	Hebdomadis	<i>kambale</i>	Kambale
Tarassovi	<i>darien</i>	637 K	Icterohaemorrhagiae	<i>mwogolo</i>	Mwogolo
Tarassovi	<i>gatuni</i>	1473 K	Icterohaemorrhagiae	<i>ndahambukuje</i>	Ndahambukuje
Tarassovi	<i>navet</i>	TRVL 109873	Icterohaemorrhagiae	<i>ndambari</i>	Ndambari
Tarassovi	<i>rama</i>	316	Pomona	<i>kunming</i>	K 5
<i>Leptospira kirschneri</i> †			Pomona	<i>mozdok</i>	5621
Australis	<i>ramisi</i>	Musa	<i>Leptospira meyeri</i> †		
Autumnalis	<i>bim</i>	1051	Ranarum	<i>ranarum</i>	ICF
Autumnalis	<i>bulgarica</i>	Nikolaev	Samaranga	<i>semaranga</i>	Veldrat Sem.173
Autumnalis	<i>butembo</i>	Butembo	<i>Leptospira biflexa</i> †		
Autumnalis	<i>erinaceauriti</i>	Erinaceus aur.670	Andamana	<i>andaman</i>	CH 11
Autumnalis	<i>lambwe</i>	Lambwe	Samaranga	<i>patoc</i>	Patoc I
Autumnalis	<i>mujunkumi</i>	EZ 237	<i>Leptospira wolbachii</i> †		
Bataviae	<i>djatzi</i>	HS 26	Codice	<i>codice</i>	CDC¶
Canicola	<i>bafani</i>	Bafani	<i>Leptospira inadai</i> †		
Canicola	<i>galtoni</i>	LT 1014	None	<i>lyme</i>	10¶
Canicola	<i>kamituga</i>	Kamituga	<i>Leptonema</i> sp.		
Canicola	<i>summeri</i>	Summer	None	<i>ilini</i>	3055¶
Cynopteri	<i>cynopteri</i>	3522 C	<i>Leptospira parva</i> †		
Djasiman	<i>agogo</i>	Agogo	None	<i>parva</i>	H¶
Grippytyphosa	<i>grippytyphosa</i>	Moskva V			
Grippytyphosa	<i>grippytyphosa</i>	Duyster§			

* All strains listed are reference strains except the strain indicated by § which is a Dutch clinical isolate.

† Species according to Yasuda *et al.* (1987) and extended by A. F. Kaufmann (personal communication).

‡ Serovars listed according to unofficially named *L. alstoni* (A. F. Kaufmann, personal communication). *L. alstoni* was replaced by *L. kirschneri* (Ramadass *et al.*, 1992).

|| Non-pathogenic leptospires according to the Subcommittee on the Taxonomy of *Leptospira*.

¶ These strains are not leptospires according to the Subcommittee on the Taxonomy of *Leptospira*.

for the detection of leptospires provided that these micro-organisms are present in relatively large numbers. With the introduction of PCR, rapid detection of small numbers of leptospires in clinical samples may become practical due to specific amplification of leptospiral DNA. Thus far, PCR has been applied to study genetic disorders but also to detect viral and bacterial infections (Demmler *et al.*, 1988; Gerritsen *et al.*, 1991; Hartskeerl *et al.*, 1989; Hookey *et al.*, 1992; Kogan *et al.*, 1987; Kolk *et al.*, 1992; Merien *et al.*, 1992; Ou *et al.*, 1988; Saiki *et al.*, 1988; de Wit *et al.*, 1991). In 1989, we developed a primer set and probes for the detection of Hardjobovis (serovar *hardjo*) in cattle urine by PCR (van Eys *et al.*, 1989). In this study we describe the selection and construction of primer sets for the specific amplification of DNA of pathogenic leptospires and we present the DNA sequence analysis of PCR products from various leptospiral strains. To explore the potential of the PCR as a tool for early diagnosis of leptospirosis, we applied this method to sera of patients with proven leptospirosis aiming at the detection of leptospires in blood during the first 10 days of disease, i.e. largely the period between the onset of symptoms and the appearance of antibodies.

Methods

Strains and plasmids. Leptospiral strains used in this study were from our Reference Collection of *Leptospira* (Table 1). Other micro-organisms used were *Borrelia burgdorferi*, *Treponema reuteri*, *Mycobacterium* spp., *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Salmonella* sp. group B, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Yersinia enterocolytica* and *Escherichia coli* (kindly provided by Dr R. J. van Ketel, Department of Medical Microbiology, Academic Medical Hospital, Amsterdam, The Netherlands). *E. coli* strain DH5α (Hanahan & Meselson, 1983) was used as a host for plasmid pUC19 (Yanisch-Perron *et al.*, 1985) and derivatives.

Culture media and reagents. Leptospires were cultured in EMJH medium as described by Johnson & Harris (1967). *E. coli* DH5α was grown either in LB medium or in SOB medium (Sambrook *et al.*, 1989). When appropriate, ampicillin, IPTG, X-gal and Difco agar (1.5%, w/v) were added to the LB medium (Sambrook *et al.*, 1989). Guanidinium thiocyanate (GuSCN) was from Fluka. Diatoms (high purity, analytical grade kieselguhr DG) were obtained from Riedel-de Haën.

Restriction enzymes and T4 DNA ligase were purchased from Boehringer. Heat-stable *Taq* DNA polymerase was from Perkin-Elmer Cetus. Solutions of deoxynucleotides dATP, dTTP, dCTP, dGTP (100 mM, pH 7.5) and dUTP (200 mM, pH 7.5) were from Pharmacia.

Collection of clinical samples and culture. All paired blood samples used in this study were from 79 leptospirosis patients in The Netherlands and on Barbados. Forty samples from patients suspected

Fig. 1. (a) Nucleotide sequence of the DNA insert of recombinant plasmid pLIP60. The positions of primers G1 and G2 are indicated. (b) Nucleotide sequence of the DNA insert of recombinant plasmid pBIM64. The positions of primers B64-I and B64-II are indicated.

of leptospirosis but serologically negative, and 40 samples from healthy individuals were used as negative control sera. Blood was clotted at room temperature and serum frozen at -20°C . Sera from 60 patients and 40 negative control sera had been collected several years before PCR was applied. These sera were kept for several weeks at 4°C for serology and later frozen at -20°C . The sera of the other 19 patients and 40 negative control sera collected over the last 2 years were directly frozen at -20°C after blood was drawn. First samples of leptospirosis patients were collected before the 10th day after onset of the disease, and second samples at least 1 week later. First as well as second samples were examined serologically. A few drops of whole blood of first samples were inoculated directly after collection into EMJH medium for culturing.

In preliminary experiments we found that red blood cells inhibited PCR. The maximum volume of whole blood which could be processed without severe inhibition of the PCR was 100 μl , whereas 1–3 ml serum could be used without any problems. The PCR assay on serum gives much better results than on whole blood samples, in spite of possible loss of leptospires trapped in blood clots. Therefore, PCR was performed on 1 ml serum of the first sample instead of blood.

Serology. To establish a titre rise or seroconversion the microscopic agglutination test (MAT) was applied according to standard method-

ology (Dikken & Kmety, 1978). A battery of live leptospiral strains recommended by Faine (1982) was used as antigens in this test. ELISA was performed as described previously (Terpstra *et al.*, 1980). Strain Wijnberg (serovar *copenhageni*) and strain Hardjoprajitno (serovar *hardjo*) were used as antigens in ELISA. High IgM levels against leptospires were considered indicative of current or recent infection.

Identification of strains. The strains isolated from humans were typed by the cross-agglutination absorption test (CAAT) (Dikken & Kmety, 1978), and by use of monoclonal antibodies in agglutination tests (Korver *et al.*, 1988; Terpstra *et al.*, 1987).

DNA technology. Standard procedures were used for DNA preparation, restriction enzyme digestion, ligation, and transformation (Sambrook *et al.*, 1989). DNA fragments were sequenced using an automated sequencing apparatus and the sequencing kit of Applied Biosystems according to the instructions of the manufacturer. DNA sequence data were processed using the PC/GENE 6.6 software (IntelliGenetics). Preparation of ^{32}P -labelled DNA probes was done with a random priming labelling kit (Boehringer). Southern blotting (Southern, 1975) and hybridization with ^{32}P -labelled DNA probes were performed according to standard procedures (Sambrook *et al.*, 1989). Non-radioactive probes were made using a DNA digoxigenin-dUTP

labelling and detection kit (Boehringer), and hybridization was done as described by Hartskeerl *et al.* (1989). Hybridization and washing steps were done at 68 °C (high stringency) or at 55 °C (low stringency).

Construction of genomic DNA libraries. Construction of a genomic DNA library of *L. interrogans* strain RGA has been described previously (van Eys *et al.*, 1991). To construct a genomic DNA library of *L. kirschneri* strain 1051, *EcoRI*-digested genomic DNA was ligated into pUC19. *E. coli* strain DH5 α was used for transformation.

Purification of DNA from clinical specimens. DNA was extracted according to Boom *et al.* (1990) with minor modifications (Gravekamp *et al.*, 1991). Serum (1 ml) was mixed with 9 vols of a GuSCN-lysis buffer (120 g GuSCN, 100 ml 0.1 M-Tris/HCl, pH 6.4, 22 ml 0.2 M-EDTA, and 2.6 ml Triton X-100). Subsequently, 40 μ l of a suspension of diatoms (10 g diatoms, 50 ml distilled water, and 500 μ l 32%, w/v, HCl) was added and the mixtures were thoroughly vortexed. The diatoms complexed with DNA were rapidly sedimented by centrifugation, washed twice with a GuSCN-containing washing-buffer (120 g GuSCN, and 100 ml 0.1 M-Tris/HCl, pH 6.4), twice with 70% (v/v) ethanol, and once with acetone, and finally dried at 56 °C for 10 min. DNA was eluted from the diatoms by incubation in distilled water for 10 min at 56 °C and subsequently treated with proteinase K (250 μ g ml⁻¹ final concentration) for 10 min. Finally, proteinase K was inactivated by incubation at 100 °C for 10 min.

PCR. Primers G1 (5' CTG AAT CGC TGT ATA AAA GT) and G2 (5' GGA AAA CAA ATG GTC GGA AG) were derived from the 5' end (nucleotides 1–20) and the 3' end (nucleotides 264–285), respectively, of the sequence of the recombinant plasmid pLIPs60 shown in Fig. 1(a). Primers B64-I (5' CTG AAT TCT CAT CTC AAC TC) and B64-II (5' GCA GAA ATC AGA TGG ACG AT) were derived from the 5' end (nucleotides 1–20) and the 3' end (nucleotides 542–563), respectively, of the sequence of recombinant plasmid pBIM64 presented in Fig. 1(b).

PCR was performed as described previously (van Eys *et al.*, 1989) with minor modifications. Briefly, 40 μ l of DNA samples were mixed with 5 μ l of the reaction buffer (10 $\times$ buffer: 500 mM-KCl, 20 mM-MgCl₂, 100 mM-Tris/HCl, pH 9.0, and 10 μ g gelatin ml⁻¹), 0.5 μ l of a 100 pM solution of each primer, 0.5 μ l of a mixture containing 25 mM of each of the four deoxynucleotides dATP, dTTP, dCTP and dGTP, 0.1 μ l *Taq* polymerase (0.5 U) and 3.4 μ l distilled water to a final volume of 50 μ l. DNA amplification reactions were performed in a Pharmacia LKB Gene ATAQ controller using 32 cycles. One amplification cycle consisted of denaturation of the DNA for 90 s at 94 °C, annealing of the primers for 60 s at 55 °C and elongation for 120 s at 72 °C.

To prevent false-positive reactions by contaminating PCR products from earlier experiments, dUTP was incorporated and PCR mixtures were treated with uracil-N-glucosylase to degrade contaminating PCR products as described by Persing (1991). Furthermore, rooms and equipment were decontaminated with Chlorox according to Prince & Andrus (1992).

Results

Screening of DNA libraries and selection of primers

Clone pLIPs60 was selected from the genomic DNA library of *L. interrogans* strain RGA on the basis of a strong hybridization signal with DNA from most *Leptospira* species as described previously (van Eys *et al.*, 1991). This clone reacted to representatives of all

pathogenic *Leptospira* species according to Yasuda *et al.* (1987) with the exception of *L. kirschneri*. The nucleotide sequence of pLIPs60 was determined (Fig. 1a) and used for the selection of primers G1 and G2.

Species *L. kirschneri* comprises important pathogenic leptospiral strains. To design a PCR assay that included the detection of leptospiral strains of this species, a second genomic DNA library was made of *L. kirschneri* strain 1051. Six selected recombinant clones, containing inserts of 200–600 bp, were further characterized. Specificity of the clones was tested by Southern blot analysis with *EcoRI*-digested genomic DNA from serovar *icterohaemorrhagiae* of species *L. interrogans*, serovar *hardjo* of species *L. borgpetersenii*, and serovars *bim*, *mozdok*, *mwogolo* and *grippotyphosa* all belonging to species *L. kirschneri*. Two recombinant plasmids, pBIM12 and pBIM64, gave a strong hybridization signal with a DNA fragment of about 600 bp from serovars of species *L. kirschneri* only (data not shown). The nucleotide sequence of these two recombinants was determined. The nucleotide sequence of pBIM64 (Fig. 1b) was used for the selection of primers B64-I and B64-II.

We have tested a large number of reference strains belonging to all pathogenic and non-pathogenic *Leptospira* species as mentioned in Table 1. Strains of all pathogenic *Leptospira* species except *L. kirschneri* (Table 1) were amplified by primers G1 and G2, as expected on the basis of hybridization experiments with plasmid pLIPs60. DNA from strains of the pathogenic species *L. kirschneri* only (Table 1) was amplified by primer set B64-I/B64-II which is in agreement with the hybridization experiments with plasmid pBIM64. The detection level of both PCRs was estimated to be 1–10 leptospires in 1 ml spiked serum (Gravekamp *et al.*, 1991). The combined primer sets G1/G2 and B64-I/B64-II amplified DNA by PCR from all pathogenic *Leptospira* species, i.e. strains of *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai* and *L. meyeri* strain ICF were amplified by G1 and G2, whereas strains of *L. kirschneri* were amplified by B64-I and B64-II. Representative examples of PCR results from all these pathogenic species and one non-pathogenic species, *L. biflexa* strain Patoc I, using the mixed primer sets are shown in Fig. 2(a). As compared to the results obtained with the PCRs using the distinct primer sets G1/G2 and B64-I/B64-II, no mutual interference reactions were observed when PCR was performed with a mixture of these primer sets (data not shown).

Amplification of DNA from *L. inadai* strain 10 and *L. meyeri* strain Veldrat Semarang 173 gave PCR products of the correct size. These PCR products did not hybridize with probes derived from the seven pathogenic species in this study (data not shown). The combined primer sets

Fig. 2. (a) Agarose gel (2%) showing PCR products of various strains from pathogenic and non-pathogenic *Leptospira* species using primers G1/G2 and B64-I/B64-II. Lanes: 1, molecular mass markers; 2, mixture of DNA from *L. interrogans* strain M20 and *L. kirschneri* strain 1051 (positive controls); 3, DNA from *L. kirschneri* strain 1051 (positive control); 4, DNA from *L. interrogans* strain M20 (positive control); 5, empty; 6, *L. kirschneri* strain Duyster; 7, *L. interrogans* strain M20; 8, *L. meyeri* strain ICF; 9, *L. biflexa* strain Patoc I; 10, *L. santarosai* strain TRVL 34056; 11, *L. weilii* strain Celledoni; 12, *L. kirschneri* strain 1051; 13, *L. noguchii* strain 1161U; 14, *L. borgpetersenii* strain Mus 127; 15, *L. interrogans* strain Jez Bratislava; and 16, molecular mass markers. DNA fragments of 285 bp are PCR products generated with G1/G2 primers, 563 bp fragments are PCR products generated with B64-I/B64-II primers. Hybridization with an amplicon DNA probe derived from strain Wijnberg under low (b) and high (c) stringency conditions and from strain 1051 under high stringency conditions (d) with PCR products of various members (see Fig. 2a) of pathogenic and non-pathogenic *Leptospira* species.

did not result in a detectable amplification of DNA from *L. wolbachii*, *L. parva* and *Leptonema illini* and from other microbiological sources (data not shown).

Specificity of the probes

To study DNA polymorphism in the region spanned by primers G1 and G2 of the various *Leptospira* species hybridization experiments were performed under high and low stringency conditions. The G1/G2-generated PCR product from *L. interrogans* strain Wijnberg, when used as probe, hybridized well to G1/G2-generated PCR products from genomic DNA of all pathogenic *Leptospira* species, except *L. kirschneri*, in a Southern blot performed under conditions of low stringency (Fig. 2b). In contrast to the low stringency conditions, Southern blot hybridizations done under high stringency conditions gave strong bands with PCR products generated from members of the species *L. interrogans* only: serovars *icterohaemorrhagiae*, *copenhageni* and *bratislava* (Fig. 2c). Although the nucleotide sequences corresponding to primers G1 and G2 are highly conserved, the DNA region spanned by these primers

apparently shows polymorphism between various *Leptospira* species.

As expected, the B64-I/B64-II-generated amplicon probe derived from DNA of serovar *bim* hybridized only to PCR products from serovars *bim* and *grippotyphosa* (Fig. 2d), which both belong to species *L. kirschneri*.

Inter-species DNA polymorphism

To investigate further the DNA polymorphisms as revealed by the hybridization experiments under different stringency conditions, the DNA sequence of regions between G1 and G2 was determined for all pathogenic species. Representative DNA sequences are shown as a multiple alignment in Fig. 3. We determined the homology between the various sequences flanked by G1 and G2 and the corresponding sequence of *L. interrogans* strain RGA. In accordance with the results obtained from the Southern blotting analysis, we found that sequences of members of species *L. interrogans* showed a high degree of homology (98–100%) with the sequence of strain RGA. The homology between sequences of other *Leptospira* species and that of strain RGA was clearly less, i.e. 65–90%.

M20	CTGAATCGCTGTATAAAAGTAAGCAAAGAATACAATTAAAGCGGTATAAA	50
Jez Bratislava	CTGAATCGCTGTATAAAAGTAAGCAAAGAATACAATTAAAGCGGTATAAA	50
TRVL 34056	CTGAATCGCTGTATAAAAGTATGCGAAGAAAATAATCAAAGAAGTATAGA	50
Celledoni	CTGAATCGCTGTATAAAAGTATGCGCGAAGAGATAATCAAAGAAGTATAGA	50
Mus 127	CTGAATCGCTGTATAAAAGTATGCGAAGAACACGATCAAAGAAGTATAGA	50
1161 U	CTGAATCGCTGTATAAAAGTAAGCAAAAAATACAATCAAAGCGGTATAAA	50
ICF	CTGAATCGCTGTATAAAAGTAGCGAAAGAAATACGATCAGAGAAGTATAAA	50
<div style="display: flex; justify-content: space-between; align-items: center;"> G1 ***** </div>		
M20	TTACGAAATAAAATAACGCATGATACCAAATCTGAGAGAATGGATTAAAA	100
Jez Bratislava	TTGCGAAATAAAATAACGCATGATACCAAATCTGAGAGAATGGATTAAAA	100
TRVL 34056	TCACAAAGTAGAATAATGCGTGATACAGATCTGCGAGAATGGATTGAAA	100
Celledoni	TTACATAGTAGAACAACCGGTGATACAGATCTGGGAGAACGGATTGAAA	100
Mus 127	TTATATAGTAGAACAATGCGATGATACAGATCTGGGAGAATGGATTGAAA	100
1161 U	TTACAAAATAAAACAAATGCGATGATACAGATTTGAGAGAATGGATTGAAA	100
ICF	TCACAAAGTAGAAGAAGTGCATGATACAGATCTGGGAGAATGGATTGAAG	100
<div style="display: flex; justify-content: space-between; align-items: center;"> * ***** </div>		
M20	AAATCCATAATCACTGCCCATCCAGCCCATTTCTGACTACTATTAGATAA	150
Jez Bratislava	AAATCCATAATCACTGCCCATCCAGCCCATTTCTGACTACTATTAGATAA	150
TRVL 34056	AAGTCCATAATGATCGCCCAACCGCCCATGTTGCGTCTGGAAGACAA	150
Celledoni	AAGTCCATAATGATCGCCCAACCGCCCATGTTGCGTCTGGAAGACAA	150
Mus 127	AAGTCCATAATGATCGCCCAACCGCCCATGTTGCGTCTGGAAGACAA	150
1161 U	AAATCCATAATCACTGCCCATCCAGCCCATTTCTGACTACTATTGGATAA	150
ICF	AAGTCCATAATCACTGCCCATCCAGCCCATTTCTGACTGCTATTGGACAA	150
<div style="display: flex; justify-content: space-between; align-items: center;"> ** ***** </div>		
M20	CCATTGAATAATCGTCTGAGGAAATAAAATCAAAGACGAAGCAAAAATGA	200
Jez Bratislava	CCATTGAATAATCGTCTGAGGAAATAAAATCAAAGACGAAGCAAAAATGA	200
TRVL 34056	CCACTGAATGATCGTCTGTGGGAACAGGATCAAGGAAGAAGCAAAGATGA	200
Celledoni	CCACTGAATGATCGTCTGTGGGAACAGGATCAAGGAAGAAGCAAAAATGA	200
Mus 127	CCACTGAATGATCGTCTGTGGGAACAGGATCAAGGAAGAAGCAAAGATGA	200
1161 U	CCATTGAATAATCGTTTGTGGGAACAAATTAAGAAGAAGCAAAGATGA	200
ICF	CCACTGATGATCGTCTGTGGGAACAAATCAAAGAAGAAGCAAAGATGA	200
<div style="display: flex; justify-content: space-between; align-items: center;"> *** ***** </div>		
M20	TCGGCATCACGTTTCGCGCCGTTTACTTTGAAAGGAATAGATTGACTCTTG	250
Jez Bratislava	TCGGCATCACGTTTCGCGCCGTTTACTTTGAAAGGAATAGATTGACTCTTG	250
TRVL 34056	TCGGCATTACGTTTCGCGCCGTTTACTTTAAAAGGAATGGATTGGCTTTTC	250
Celledoni	TCGGCATTACGTTTTCGCGCCGTTTACTTTAAAAGGAATGGATTGGCTTTTC	250
Mus 127	TCGGCATTACGTTTTCGCGCCGTTTACTTTAAAAGGAATAGATTGGCTTTTC	250
1161 U	TCGGCATCACGTTTTCGCGCCGTTTACTTTGAAAGGAATCGATTGGCTTTTC	250
ICF	TCGGCATAACGTTTCGCGCCGTTTACTTTGAAAGGAATGGACTTGGCTTTTC	250
<div style="display: flex; justify-content: space-between; align-items: center;"> ***** ***** </div>		
M20	GCCTGAACCATTTTCTTCCGACCATTTGTTTTCC (99.6%)	285
Jez Bratislava	GCCTGAACCATTTTCTTCCGACCATTTGTTTTCC (98.4%)	285
TRVL 34056	GCCTGAACCATTTTCTTCCGACCATTTGTTTTCC (79.6%)	285
Celledoni	GCCTGAACCATTTTCTTCCGACCATTTGTTTTCC (79.6%)	285
Mus 127	GCCTGAACCATTTTCTTCCGACCATTTGTTTTCC (80.8%)	285
1161 U	GCTTGAACCATTTTCTTCCGACCATTTGTTTTCC (90.2%)	285
ICF	GCCTGACCATTTTCTTCCGACCATTTGTTTTCC (82.4%)	285
<div style="display: flex; justify-content: space-between; align-items: center;"> ** ***** </div>		
G2		

Fig. 3. Multiple sequence alignment of PCR products obtained with primer set G1/G2 from members of all pathogenic species, with the exception of *L. kirschneri* which was not amplified by G1/G2. *L. interrogans* strain M20; *L. interrogans* strain Jez Bratislava; *L. santarosai* strain TRVL 34056; *L. weilii* strain Celledoni; *L. borgpetersenii* strain Mus 127; *L. noguchii* strain 1161 U; *L. meyeri* strain ICF. As indicated, sequences at the 5' end (nucleotides 1–20) and the 3' end (nucleotides 266–285) correspond to the primers G1 and G2, respectively. Percentages of homology between the sequences flanked by G1 and G2 and the corresponding sequence of *L. interrogans* strain RGA, as depicted in Fig. 1(a), are shown in parentheses. Asterisks are used to show that a position in the alignment is perfectly conserved.

Table 2. Comparison of PCR with cultures

Culture was performed on blood and PCR on serum (see Methods). All sera were tested two or more times by PCR and gave reproducible results. PCR positive, DNA bands were visible on Southern blots. No DNA bands were visible on Southern blots in 80 negative controls (40 samples from patients without leptospirosis and 40 samples from healthy individuals).

	PCR positive	PCR negative	Total
Culture positive	20	8	28
Culture negative	19	32	51
Total	39	40	79

PCR on serum samples

Table 2 shows that PCR (50%; 39/79) was more often positive than culturing (35%; 28/79) on samples from proven leptospirosis patients. Still both methods failed in

more than one-third (40%; 32/79) of the cases and there was a considerable discrepancy between results of both methods. About 70% (20/28) of the serum samples from which *icterohaemorrhagiae/copenhageni*, *bim* or *grippotyphosa* could be isolated by culturing was positive by PCR (Table 3). We demonstrated the presence of leptospires by PCR at a comparable rate of about 60% (16–27) of the samples from patients infected with *icterohaemorrhagiae/copenhageni* or *hardjo* from which no leptospires could be cultured. For culturally negative serum samples from patients with a *grippotyphosa* infection or *pomona* infection, this rate was 12% (3/24). All leptospirosis patients showed high IgM levels by ELISA and a titre rise or seroconversion in MAT in second samples (results not shown). Forty sera from patients without leptospirosis and 40 sera from healthy individuals used as a negative control gave negative results by PCR and Southern blotting.

Table 3. Comparison of PCR with cultures

Culture was performed on blood and PCR on serum (see Methods). All sera were tested two or more times by PCR and gave reproducible results. PCR positive, DNA bands were visible on Southern blots. No DNA bands were visible on Southern blots in 80 negative controls (40 samples from patients without leptospirosis and 40 samples from healthy individuals).

Serovar	Species	PCR positive/ culture positive	PCR positive/ culture negative
<i>icterohaemorrhagiae/copenhageni</i>	<i>L. interrogans</i>	9/12	8/12
<i>hardjo</i>	<i>L. borgpetersenii</i>	0/0	8/15
<i>grippotyphosa</i>	<i>L. kirschneri</i>	3/4	2/16
<i>bim</i>	<i>L. kirschneri</i>	8/12	0/0
<i>pomona</i>	<i>L. interrogans</i>	0/0	1/8

Discussion

In this study amplification by PCR of DNA from practically all reference strains of all presently recognized pathogenic *Leptospira* species was evaluated. A PCR using two sets of primers, G1/G2 and B64-I/B64-II, amplified DNA from serovars belonging to all pathogenic *Leptospira* species as described by Yasuda *et al.* (1987) and Ramadass *et al.* (1992). Our results do not concur completely with those obtained by PCR performed on the DNA sequence of the 16S rRNA gene (Hookey, 1992). Hookey (1992) found PCR products of the correct size from DNA of both strains ICF and Veldrat Semarang 173 of *L. meyeri*, described as a non-pathogenic species (Yasuda *et al.*, 1987). We found an anomalous PCR product from strain Veldrat Semarang 173, whereas strain ICF gave a PCR product of the correct size which hybridized well with pathogenic-*Leptospira*-specific probes. Therefore, the designation by Yasuda *et al.* (1987) of all strains of species *L. meyeri* to a non-pathogenic status is questionable. Our results and also those described by Segers *et al.* (1991) were in accordance with the classification of both strains by the International Committee on Systematic Bacteriology, Subcommittee on Taxonomy of *Leptospira*, which consider strain ICF as pathogenic and strain Veldrat Semarang 173 as non-pathogenic. In the case of strain 10, the only strain of species *L. inadai*, we found an anomalous PCR product, whereas Hookey (1992) found a PCR product of the correct size. *L. inadai* strain 10 was reported by Yasuda *et al.* (1987) as a pathogenic *Leptospira* species. However, our result suggests the need for further investigation. The anomalous PCR products of strain Veldrat Semarang 173 and strain 10 are probably due to mispriming events. They do not interfere with the specific detection of DNA from pathogenic leptospires in clinical serum samples because they do not hybridize with the *Leptospira*-specific probes. The 16S rRNA gene is highly conserved. Primers derived from this gene in pathogenic leptospires may still amplify in

PCR DNA of other micro-organisms such as *E. coli* and *Actinomyces pyogenes* (Wagenaar *et al.*, 1991) or non-pathogenic *L. biflexa* Patoc I (Merien *et al.*, 1992).

Inter-species DNA polymorphisms within the genus *Leptospira* have been demonstrated by Yasuda *et al.* (1987), Ramadass *et al.* (1992) and Herrmann *et al.* (1992). Our results obtained by PCR, hybridization and DNA sequencing give further and more detailed evidence of polymorphism such as observed between ICF and Veldrat Semarang 173.

The results obtained by PCR applied on clinical samples depend greatly on the purity of the extracted target DNA. The presence of inhibitory factors in serum samples which impede amplification reactions have to be taken into account. Extraction of DNA using diatom/GuSCN as described by Boom *et al.* (1990) has been demonstrated to result in a sensitivity of 1–10 leptospires (ml spiked serum samples)⁻¹ (Gravekamp *et al.*, 1991). These results correspond fairly well to those described by Burstain *et al.* (1991) and Hay *et al.* (1990) who detected 1–10 treponemes (50 µl)⁻¹ by PCR, and Noordhoek (1991) who detected 100 treponemes in 1 ml cerebrospinal fluid by PCR.

In this study we have shown the practical value of the PCR for the early diagnosis of leptospirosis when we extracted DNA from serum of patients infected by prevalent serovars in The Netherlands and Barbados with the diatoms/GuSCN method. Comparison of results obtained by PCR to those obtained by culturing indicates that PCR is more sensitive. Failure of leptospires to grow in culture is generally observed and may, at least partly, be attributed to fastidiousness of leptospires. It is not clear why PCR failed in the detection of leptospiral DNA in some sera of proven patients. This failure is possibly due to the fact that most of sera used in this study had been stored for considerable time at 4 °C, and subsequently on several occasions frozen and thawed which may have led to DNA degradation. In addition, PCR may fail when leptospires are present in very low numbers below the detection level

of 1–10 leptospires ml⁻¹. It is remarkable that in severe infections with *icterohaemorrhagiae/copenhageni* and *bim* both PCR and culture were more often positive than in generally mild infections such as *pomona*, *hardjo* and *grippotyphosa*. We postulate that a lower number of leptospires in the blood of patients with mild infections is a cause of lower yields by both PCR and culture. Testing larger volumes of sera may yield a higher positive score by PCR. The cause or causes of PCR failures on serum from proven patients need to be clarified and, if possible, removed.

As reported by Persing (1991), the occurrence of false positive PCR results is caused rather by contamination with amplified DNA than by contamination with target DNA. In accordance with this report, we found that all negative control sera, collected in the same period as the patients' sera, were negative by PCR.

We conclude that PCR is a promising method for the quick and early detection of leptospires in clinical samples in the period between the first appearance of clinical symptoms and when antibodies become detectable. This study is complementary to our earlier studies on urine (van Eys *et al.*, 1989) and studies on urine by others (Gerritsen *et al.*, 1991; Merien *et al.*, 1992), and studies on plasma and cerebrospinal fluid (Merien *et al.*, 1992). The DNA polymorphism in PCR products deserves to be explored for the identification of causative species or even strains.

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