Detection of Leptospiraceae by amplification of 16S ribosomal DNA

J.V. Hooke

PHLS Leptospira Reference Laboratory, FAO/WHO Collaborating Centre, County Hospital, Hereford, U.K.

Received 10 October 1991
Revision received 4 November 1991
Accepted 5 November 1991

Key words: Leptospiraceae; Polymerase chain reaction; 16S rDNA

1. SUMMARY

The polymerase chain reaction (PCR) was developed to detect Leptospiraceae. Primers were used to amplify a 631 base-pair (bp) 5’-region of 16S rDNA. Representative strains from the species, Leptospira interrogaens sensu stricto, L. borgpetersenii, L. noguchii, L. santarosai, L. weilii, L. inadai, L. meyeri and the single member strain of Leptonema were amplified. In contrast, strains representing the saprophytic species, L. biflexa, L. wolbachii and L. parva were not amplified. There was no PCR product from 23 phylogenetically unrelated species of bacteria. As little as 10–1 pg of purified DNA and as few as 10–1 leptospires could be detected using this PCR analysis. Isolates of leptospires from clinical sources gave a positive PCR band, but those from surface waters did not.

2. INTRODUCTION


The detection of leptospires (Leptospira and Leptonema) in clinical and environmental samples remains limited and difficult. Conventional methods such as isolation by culture and subsequent detection by dark-field microscopy are either too slow (up to 3 months) or unreliable [3]. Chromosomal DNA probes have been developed [4] yet they were neither specific nor sensitive (between 750 and 2500 organisms). An alternative approach is to use probes that are complementary to small ribosomal subunits (16S rRNAs). Oligonucleotide primers for use in the polymerase chain reaction (PCR) and complementary to defined regions of the 16S rRNA gene have been used in the detection of mycobacteria [5].
The aim of this study was to develop a rapid, specific and sensitive method for detecting Leptospiraceae based on the amplification of 16S rDNA.

3. MATERIALS AND METHODS

3.1. Bacterial strains and culture procedures

The strains used are given in the legends to Figs. 1 and 2. Strains of *Leptospira* and isolates from clinical and environmental origins were cultured according to Ellinghausen and McCullough [6].

3.2. DNA preparation

Chromosomal DNA was isolated and purified as previously described [7].

3.3. Preparation of biotin labelled 16S ribosomal DNA

Plasmid DNA (plasmid pNO1311 contains a 734-bp 5'-3' HindIII-AvalI 16S rRNA gene fragment insert derived from pKK 3535 [8]) was extracted from cultured *Escherichia coli* NO2356 host cells according to the manufacturer's instructions (Circleprep™, Stratagene, CA) and 100 ng of DNA labelled using a random-primer kit in the presence of biotin-11-dUTP following the protocol given by the manufacturer's (Gibco BRL, Paisley, Scotland).

3.4. Amplification of 16S ribosomal DNA by PCR

PCR was performed on a Perkin-Elmer Thermal Cycler (Perkin-Elmer Limited, Buckinghamshire HP9 IQA, U.K.) in PCR tubes that contained approximately 100 ng of DNA template and the reaction mix made up of 5 μl of 10× PCR reaction buffer (500 mM KCl, 100 mM Tris·HCl, pH 8.3, 3.5 mM MgCl₂, 0.01% gelatin), 100 μM each deoxynucleotide triphosphate (Pharmacia, Milton Keynes MK5 8PH, U.K.), 50 pM each primer and the sequences for these were:

- 5’ CGC TGG CGG GCC GTC TTA AA 3’ (34...53) and
- 3’ AAG GTC CAA ATC GCC ACT T 5’ (647...665)

glycerol at 11% (v/v) (Gibco BRL), 1.25 units of ‘AmpliTaq’ DNA polymerase (I.L.S. Limited, London EC1A 7HU, U.K.) and sterile ‘Millipure’ water to 50 μl.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.

Following an initial 5-min incubation at 95°C, thermal cycling (× 32 cycles) proceeded with each segment of one cycle being: 94°C for 40 s; 58°C for 60 s; and 72°C for 60 s.
for 17 s; and 72°C for 1 min. An additional cycle at 72°C for 10 min was included as a final step.

3.5. Analysis of amplified rDNA

5 or 10 µl of product were taken for analysis by gel electrophoresis (3.0% NuSieve GTG/SeaKem GTG composite agarose Tris-acetate-EDTA, pH 8.0 buffered gel) at 100 mA for 2 h. Gels were stained, visualised and photographed as previously described [7].

Gels were blotted onto nylon membranes (0.45 µm pore size ‘Hybond N’; Amersham International, Buckinghamshire HP7 9LL, U.K.) in the presence of 20 × SSPE (saline-sodium phosphate-
Fig. 2. Agarose gel analysis of PCR-amplified product from clinical and environmental leptospire isolates and from phylogenetically unrelated sources. Lanes 1 and 21 were GYJ74 RF DNA-HaeIII fragment size-markers; lane 2, *L. interrogans* sensu stricto ICTEROHAEMORRHAGIAE; *icterohaemorrhagiae* RGA (source Amsterdam and included in this assay as a weak-positive control); lanes 3 and 4 were clinical isolates; and lanes 5–9 were environmental leptospire isolates; lane 10, *Borrelia burgdorferi* strain B31; ATCC *15210; lane 11, *Campylobacter cimaedi* NCTC *+ + 11959; 12, *C. coli* NCTC 11351; 13, *C. divergens* NCTC 11885; 14, *C. felicis* NCTC 11613; 15, *C. fetus* ssp. *fetus* NCTC 10442; 16, *C. fetus* NCTC 11351; 17, *C. jejuni* NCTC 11392; 18, *C. mucosalis* NCTC 11001; 19, *Helicobacter pylori* NCTC 11638; 20, *H. pylori* NCTC 11916; 22, *Escherichia coli* NCTC 9001; 23, *Haemophilus influenzae* (wild strain); 24, *Klebsiella pneumoniae*; 25, *Legionella micdadei* strain TATLOCK; 26, *L. pneumophila* NCTC 1192; 27, *Listeria monocytogenes* (wild strain); 28, *Mycobacterium tuberculosis*; 29, *M. bovis* (wild strain); 30, *M. bovis* BCG; 31, *Pseudomonas aeruginosa*; 32, *Salmonella typhimurium*; 33, *Staphylococcus aureus*; 34, *Streptococcus pneumoniae* NCTC 7978; 35, *Yersinia enterocolitica* NCTC 11177; 36, *Wolinella recta* NCTC 11489; 37, *Succinogluconates eutrophus*; 38, herring sperm DNA; 39, human DNA; and 40, a negative control having no DNA template. 5 μl of amplified product were loaded to each well. * American Type Culture Collection; ** National Collection of Type Cultures.
Fig. 3. Agarose gel analysis of PCR-amplified product from *L. interrogans sensu stricto* ICTEROHAEMORRHAGIAE *icterohaemorrhagiae* RGA (source Amsterdam). Lanes 1 and 11 were OX174 RF DNA-HaeIII and lanes 10 and 20 were biotin-OX174 RF DNA-HinIII fragment size-markers. Lanes 2–8 were serial 10-fold dilutions of purified-DNA; and were 100 ng (lane 2), 10 ng (lane 3), 1 ng (lane 4), 100 pg (lane 5), 10 pg (lane 6), 1 pg (lane 7), 100 fg (lane 8) and lane 9 was a negative control having no DNA-template. Lanes 12–18 were serial 10-fold dilutions of a suspension of *L. interrogans* RGA containing $1 \times 10^5$ bacteria ml$^{-1}$ (lane 12), $1 \times 10^4$ (lane 13), $1 \times 10^5$ (lane 14), $1 \times 10^2$ (lane 15), $1 \times 10^3$ (lane 16), $1 \times 10^4$ (lane 17) and $1 \times 10^{-1}$ (lane 18) and lane 19 was a negative control having no DNA-template. 10 μl of amplified product were loaded to each well.
EDTA, pH 7.4) according to standard methods [9]. Pre-hybridisation, hybridisation and post-hybridisation conditions followed that given by Owen, Costas and Dawson [10]. Hybrids were visualised using a colourimetric non-radioactive detection kit (BluGENE™) according to the manufacturer’s instructions (Gibco BRL).

4. RESULTS AND DISCUSSION

Leptospirosis and its aetiological zoonotic agent, *L. interrogans sensu lato* [11], have been the subject of recent media interest with the apparent risk to those that come into contact with potentially contaminated waters, such as canoeists and other recreational water users. The development of a rapid, specific and sensitive method for leptospire detection in clinical and environmental samples would be of benefit in disease surveillance and epidemiological investigations.

PCR primers were chosen on the basis of published, albeit limited, 16S rRNA sequence [12] and catalog data [13]. As expected, a positive 631-bp PCR product of predicted size [12] was observed for strains representing the species *L. interrogans sensu stricto* (Fig. 1, lanes 2–14), *L. borgpetersenii* (Fig. 1, lanes 15–18), *L. noguchii* (Fig. 1, lanes 19 and 20), *L. santarosai* (Fig. 1, lanes 22–25), *L. weilii* (Fig. 1, lanes 26 and 27) and *Leptovena illini* (Fig. 1, lane 37) and a negative result was recorded for duplicate strains of *L. biflexa SEMARANGA patoc* Patoc 1 (Fig. 1, lanes 28 and 29). Since *L. biflexa* is the nucleus for saprophytic strains [2] it was not unexpected to observe that a negative result was also obtained for the duplicate and closely related strain, *L. biflexa ANDAMANA andamana* CH 11 (Fig. 1, lanes 30 and 31).

A faint and anomalous ‘ghost’-product of approximately 450 bp in size was visualised on agarose-gels and Southern blots (Figs. 1, 2 and 3). This result indicates that mispriming events were occurring within the target, yet it does not present a problem in this analysis since this band was not of the expected or even correct size.

Other species of *Leptospira* were also analysed. Negative results were obtained for the saprophytic forms, *L. wolbachii CODICE cdc* Biflexa CDC (Fig. 1, lane 34) and *L. parva* (Fig. 1, lane 36). However, a positive 16S rDNA product was obtained from the single and pathogenic member of *L. inadai* (Fig. 1, lane 35) and from representative strains of *L. meyeri* (Fig. 1, lanes 32 and 33). The strains *RANARUM ranarum* ICF and *SEMARANGA semaranga* Veldrat S.173 were isolated from a leopard frog (*Rana pipiens*) in Iowa, U.S.A. [14] and from a rat (*Rattus brevicaulatus*) in Java [15], respectively. These strains are now regarded as being saprophytes on the sole basis of avirulence, however their physiological characteristics are that of both pathogenic and saprophytic forms [2,15]. Their true affiliation and the significance of these PCR results is, therefore unclear.

There was no amplification product from phylogenetically unrelated bacteria or from the yeast, *Saccharomyces cerevisiae*, herring sperm DNA or even human DNA (Fig. 2), indicating that this PCR analysis may be leptospire-specific.

The sensitivity was between 10 and 1 pg of purified DNA and between 10 and 1 leptospires could be detected (Fig. 3). These limits of detection are a considerable improvement on that previously obtained using DNA probes [4].

Leptospires isolated from clinical and environmental sources were studied. The clinical isolates, identified as *L. interrogans* [16], gave a positive amplification product (Fig. 2, lanes 3 and 4), yet strains isolated from surface waters did not (Fig. 2, lanes 5–9). These environmental strains have provisionally been typed, using serological methods, as being similar to *L. biflexa* (unpublished data).

With the notable exceptions of strains representing the saprophytes, *L. biflexa*, *L. wolbachii* and *L. parva*, evidence has been provided that selected primers for use in the PCR can be used in the rapid (2 days from biomass to the analysis of a PCR product), specific and sensitive detection of Leptospiroaceae and leptospire isolates.

ACKNOWLEDGEMENTS

The author is indebted to colleagues who kindly provided cultures and chromosomal DNA
and to Masayasu Normura, University of California, Irvine, U.S.A. for supplying the vector carrying the plasmid pN01311.

REFERENCES