DNA Hybridization with Hardjobovis-specific Recombinant Probes as a Method for Type Discrimination of Leptospira interrogans Serovar hardjo

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Restriction endonuclease analysis of DNA of Leptospira interrogans, serovar hardjo, showed two distinct types within this serovar. These two types, hardjoprajitno and hardjobovis, cannot be differentiated by monoclonal antibodies. Application of 32P- or biotin-labelled total DNA probes in dot-blot or in situ hybridization assays showed a high sensitivity of the assays but also considerable cross-hybridization. Therefore, a genomic library of hardjobovis was constructed and a number of hardjobovis-specific recombinant clones were isolated. Finally, four clones were selected on the basis of a strong hybridization signal and a high specificity for hardjobovis as compared to hardjoprajitno. In a dot-blot assay as well as in in situ hybridization experiments all four clones gave strong signals, and no cross-hybridization with hardjoprajitno was observed in either type of assay. Our results indicate that specific recombinant DNA probes might provide tools for routine diagnosis and classification in cases of hardjo infections.

INTRODUCTION

Detection and characterization of Leptospira is currently done with culturing and immunological techniques. Culturing is time consuming. Immunological techniques have become more rapid and specific since the availability of monoclonal antibodies (Terpstra et al., 1985), which can be used to characterize isolates to the serovar level. In most cases this is an acceptable degree of classification. However, in the case of serovar hardjo this method is not satisfactory, since within this serovar two types, hardjobovis and hardjoprajitno, occur (Thiermann & Ellis, 1986). The clinical symptoms and epidemiology of these two types differ widely (Ellis, 1986; Thiermann & Ellis, 1986). Therefore, several investigators have studied the possibilities of DNA-based techniques for the characterization of leptospiral strains.

Restriction endonuclease analysis (Marshall et al., 1981; Robinson et al., 1982; Thiermann et al., 1985, 1986) and DNA hybridization (Terpstra et al., 1986, 1987) have shown the potential of DNA-based techniques. However, the published assays have a number of shortcomings. Restriction endonuclease analysis still requires culturing of the organisms, and differences in the complex restriction pattern do not necessarily reflect differences in the biochemistry of the organism. DNA hybridization with total DNA probes is not specific enough to differentiate between leptospiral strains or types. Southern blotting with specific probes is the method of choice for the classification of Leptospira, since this method provides a particular banding pattern in addition to the specific hybridization (G. J. J. M. Van Eys & J. Zaal, unpublished results). However, the method is cumbersome and unsuitable for routine application. Techniques like dot-blotting and in situ hybridization seem the most promising for routine identification of leptospiral isolates (Terpstra et al., 1986, 1987). A dot-blot assay, with specific recombinant probes, will provide a rapid and technically simple identification system, especially suitable for processing a large number of samples. In situ hybridization, on the other hand, offers the advantage of recognizable morphology in combination with a specific
hybridization signal. This will not only facilitate detection but also at once characterize the infecting organism.

The aim of this study was to select a number of recombinant DNA clones that might differentiate between two types of *Leptospira interrogans*, the types hardjobovis and hardjoprajitno of the serovar hardjo. Probes made from such recombinant clones could be used in dot-blot and *in situ* hybridization assays.

**METHODS**

*Leptospiral strains.* For the hardjoprajitno type we used the reference strain hardjoprajitno and one cattle isolate from Northern Ireland (kindly provided by Dr W. A. Ellis); for the hardjobovis type we used one human isolate from the Netherlands, and several cattle isolates from the Netherlands, Northern Ireland (kindly provided by Dr Ellis) and the USA (kindly provided by Dr A. B. Thiermann); two untyped hardjo strains from Germany (from Dr S. Bremm) were also used. All these isolates belong to the serovar *hardjo* and were typed by restriction endonuclease analysis. Several other reference strains and field isolates were used, as indicated in the legend to Fig. 4. Culture conditions were essentially as described by Johnson & Harris (1967).

Preparation of chromosomal DNA. Exponentially growing leptospiral cultures were centrifuged at 12000 g for 30 min at 4 °C. The pellet was washed twice in phosphate-buffered saline (PBS: 0.15 m-NaCl, 6.7 mM-sodium phosphate, pH 7.2), then resuspended in lysis buffer (50 mM-NaCl, 10 mM-EDTA, 50 mM-Tris/HCl, pH 7.4). SDS and pronase (Sigma) were added to final concentrations of 0.5% and 100 µg ml⁻¹ respectively, and the mixture was incubated for 2 h at 60 °C. Proteinase K (Sigma) was then added to a concentration of 20 µg ml⁻¹ and the protein digestion was continued for another 2 h at 37 °C. The DNA solution was subjected to phenol and chloroform extraction twice and precipitated with ethanol (Maniatis et al., 1982). After two washes in 70% (v/v) ethanol the DNA was dried and redissolved in ultrapure water. It was then ready for restriction digestion by endonucleases or any other treatment.

Restriction endonuclease analysis. Chromosomal DNA was digested with endonucleases purchased from Boehringer Mannheim and New England Biolabs. The enzymes were used as recommended by the manufacturer. Digestions were performed for 2 h at 37 °C using 3 U enzyme per µg DNA, in the presence of 20 µg heat-inactivated RNAase (Worthington) ml⁻¹.

Molecular cloning. Purified chromosomal DNA of *L. interrogans* serovar *hardjo*, type hardjobovis (isolate HB013, USA cattle isolate provided by Dr A. B. Thiermann; Thiermann et al., 1986), was partially digested with HpaII (Boehringer Mannheim), and the DNA fragments were ligated in the site of the polylinker of the plasmid pUC19 (Yanisch-Perron et al., 1985). The resulting recombinant plasmids were transformed into *Escherichia coli* HB101 and the cells were plated onto nitrocellulose-covered plates. Replicas of these filters were made, processed and hybridized with ³²P-labelled chromosomal DNA of hardjoprajitno and isolate HB013. Hardjobovis-specific clones were picked and a second screening was done by dot-blot analysis. All procedures were performed according to Maniatis et al. (1982).

Southern blotting. After endonuclease digestion of chromosomal DNA, the fragments were separated by gel electrophoresis in 0.7% agarose gels in 0.4 M-Tris/0.2 M-acetate buffer, pH 8.0. When electrophoresis was completed, the gels were irradiated with UV light for 5 min and the DNA was denatured by 45 min treatment with 1 M-NaOH. The DNA was then transferred to nylon filters (Biotrans, ICN) by capillary blotting for 16 h in 20 × SSC (Southern, 1975). The filters were neutralized (3 M-NaCl, 1 M-Tris, pH 7-4), rinsed in 3 × SSC and subsequently baked for 2 h at 80 °C. Prehybridization and hybridization were performed at 68 °C in a solution containing 7% (w/v) SDS, 1% (w/v) BSA, 2 M-EDTA, 0.5 mg salmon sperm DNA ml⁻¹ and 0.5 M-Na₂HPO₄/H₃PO₄ (pH 7.0). Probe DNA was labelled to a specific activity of at least 10⁸ c.p.m. per µg DNA and 10⁻⁷ c.p.m. was added to the hybridization solution. After hybridization, the filters were washed in 3 × SSC, 0.1 × SSC and 0.1 × SSC/0.1% SDS for 1 h each. Autoradiography was performed at −70 °C on Kodak XRA film using intensifying screens. For dot-blot analysis DNA was spotted on nitrocellulose BA85 filters (Schleicher and Schuell) using a Minifold apparatus (Schleicher and Schuell). Hybridization conditions and procedures were as described for Southern blots.

*In situ* hybridization. This was done on cultured leptospiras. The bacteria were layered on microscope slides, air-dried and fixed in anhydrous methanol for 10 min. Material preparation, hybridization conditions and procedures, and preparation of biotin-labelled probes have been described previously (Terpstra et al., 1987; Van Eys et al., 1987). An additional step was added to the staining procedure. The signal of the diaminobenzidine stain was enhanced by silver accumulation (Amersham). The slides were examined by light microscopy.

**RESULTS**

Restriction endonuclease analysis with a few enzymes showed the clear difference in the genomic make-up of the two types (Fig. 1). From the genomic library that was constructed by
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Fig. 1. Restriction endonuclease analysis of hardjobovis (bo) and hardjoprajitno (pr) DNA. DNA of both leptospiral types was digested with several enzymes (B, BamHI; E, EcoRI; H, HindIII; P, PstI; BII, BglII) and fractionated on a 0.7% agarose gel.

ligation of partially digested hardjobovis DNA into plasmid pUC19, 120 clones were selected by differential screening with total DNA probes of hardjobovis and hardjoprajitno. After two more rounds of screening, four clones were selected for further analysis, on the basis of specificity and intensity of the signal.

Restriction endonuclease analysis of the four clones with a number of enzymes indicated that they were different parts of the hardjobovis genome, since no similarities in the restriction pattern were found (Fig. 2). This result was confirmed by Southern blotting. Southern blots of hardjobovis DNA showed that hybridization with clone 8645 resulted in a rather simple pattern, which conformed to the restriction pattern of the clone (Fig. 3). This could indicate that only one copy of this fragment is present in the hardjobovis genome. However, the strength of the hybridization signal in dot-blot assays and in situ hybridization was more indicative of the presence of multiple copies. Clones 8611 and 8656 appeared to be present in multiple copies whereas clone 8633 showed rather weak hybridization with a number of other fragments in addition to the hybridization expected on the basis of the restriction pattern of this clone.

Application of the recombinant probes in a dot-blot assay showed that in such a system they could differentiate between hardjobovis and hardjoprajitno. All the hardjobovis isolates were identified whereas no hybridization occurred with hardjoprajitno samples (Fig. 4a). Since the aim of screening the library was to isolate clones that could differentiate between the two hardjo types, cross-hybridization with other leptospiral serovars was not extensively investigated. The limited survey done indicated that cross-reactivity with other serovars, especially those belonging to the serogroups Sejroe and Ballum, occurred (Fig. 4a). Testing of two new isolates with the recombinant probes clearly showed that these isolates belong to the hardjobovis type (Fig. 4b). This result was confirmed by restriction endonuclease analysis.
Fig. 2. Restriction endonuclease maps of the four clones selected on the basis of intensity of hybridization signal and specificity for hardjobovis as compared to hardjoprajitno. The leptospiral DNA (solid lines) is carried by the plasmid pUC19 (dashed lines). The polylinker of the plasmid is represented by the thick black lines. B, BamHI; E, EcoRI; H, HindIII; P, PstI; X, XbaI.

Fig. 3. Southern blot analysis of hardjobovis genomic DNA hybridized to clones 8611, 8633, 8645 and 8656. Hardjobovis DNA was digested with BamHI (B), EcoRI (E), and HindIII (H), then fractionated on a 0.7% agarose gel and blotted. Identical blots were hybridized to the four recombinant clones. Loads were 4 μg DNA per lane.
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Fig. 4. (a) Dot-blot analysis of several serovars of L. interrogans. For each dot approximately 0.2 µg DNA was spotted. A1, hardjobovis (human isolate, the Netherlands); A2, hardjoprajitno (reference strain: r.s.); A3, hardjobovis (cattle isolate, the Netherlands); A4, hardjobovis (cattle isolate, USA); A5, hardjoprajitno (cattle isolate, Ireland); A6, hardjobovis (HB013, cattle isolate, USA); A7, hardjobovis (cattle isolate, N. Ireland); A8, hardjobovis (cattle isolate, the Netherlands); B1, guangdong (r.s.); B2, lora (pig isolate, Belgium); B3, bratislava (r.s.); B4, australis (r.s.); B5, lora (r.s.); B6, jalna (r.s.); B7, canicola (r.s.); B8, ballum (r.s.); C1, romanica (r.s.); C2, grippotyphosa (r.s.); C3, hardjobovis (cattle isolate, the Netherlands); C4, pomona (r.s.); C5, hebdomadis (r.s.); C6, balcanica (r.s.); C7, copenhageni (r.s.); C8, bratislava (r.s.); D1, wolfii (r.s.); D2, mini (r.s.); D3, saxkoebing (r.s.); D4, sejroe (r.s.); D5, polonica (r.s.); D6, lora (horse isolate, the Netherlands).

(b) Dot-blot of two hardjo isolates, J-I and J-II, not previously characterized (from Dr S. Bremm). DNA of cultures was isolated and spotted on nitrocellulose in a 1:3 dilution series. The first dot contains approximately 0.5 µg DNA. DNA of the reference hardjoprajitno strain (p) and the hardjobovis strain HB013 (b) were used as references (dots below the dashed lines).

In situ hybridization with total DNA probes cannot differentiate between hardjobovis and hardjoprajitno (Fig. 5a, b). In contrast, the recombinant probes hybridized with hardjobovis but no detectable staining was found on hardjoprajitno preparations (Fig. 5c–f). Although the signal provided by total DNA probes is stronger than that provided by the recombinant probes, the latter signal is still more than adequate for the detection of hardjobovis.
Fig. 5. *In situ* hybridization with hardjoprajitno and hardjobovis organisms, using biotin-labelled total DNA (a, b) and recombinant hardjobovis probes (c–f). Total DNA of hardjobovis was applied as a probe to known hardjobovis (a) and hardjoprajitno (b). Recombinant clones 8645 (c, d) and 8611 (e, f) were biotin-labelled by nick-translation and hybridized to hardjobovis (c, e) and hardjoprajitno (d, f) leptospira.

**DISCUSSION**

We have chosen hardjoprajitno and hardjobovis to investigate the possibilities of DNA-based techniques for routine identification of leptospiral isolates. Previous studies on restriction endonuclease analysis (Marshall et al., 1981; Robinson et al., 1982; Thiermann et al., 1985, 1986) and on DNA hybridization (Terpstra et al., 1986, 1987) indicated the potential of DNA-based techniques. Restriction endonuclease analysis with a number of enzymes showed clear differences between the two *hardjo* types, and our results agreed completely with those of Thiermann et al. (1985, 1986). However, we considered this method too slow and cumbersome for routine identification. In addition, minute mutations in the genome, that do not change the biochemistry of the organism, can change the restriction pattern. In this way geographical or other variants may occur.
DNA hybridization with total DNA probes showed strong hybridization but also considerable cross-hybridization, in both dot-blot and in situ hybridization assays, between several strains of Leptospira (Terpstra et al., 1986, 1987). Although total DNA probes are not suitable for classification, they still might be valuable for the detection of leptospiral infections, because of their high signal-to-noise ratio. The four clones we selected from the hardjobovis genomic library are highly specific for hardjobovis as compared to hardjoprajitno. Cross-hybridization was below the detection level in both dot-blot and in situ hybridization assays. Although the signal-to-noise ratio was lower than for total DNA probes, the recombinant probes still provided a strong signal. There were only minor differences between the recombinant probes in intensity of the hybridization signal. This is surprising considering the apparent differences in copy number suggested by Southern blotting. Clones 8611 and 8656 may represent repeated sequences with more than 10 copies per genome. In contrast, clone 8645 seems to contain a single-copy stretch of DNA, or a number of copies in tandem repeat. This might explain the intensity of the hybridization signal observed for this clone. Clone 8633 showed a much lower hybridization signal, although probes derived from it had the same specific activity as those derived from the other clones. The significance of the (weaker) bands that are observed in addition to those expected on the basis of the restriction pattern of the clone is not clear. Repeated DNA sequences have been described in other prokaryotes (McPheat & McNally, 1987; Stern et al., 1984; Kleckner, 1981). Further research into the nature of the selected clones will be needed to confirm the present findings.

The use of specific recombinant DNA probes greatly increases the potential and the applicability of DNA hybridization techniques. The sensitivity of the hybridization techniques and the specificity of the recombinant probes allow detection and classification of the infecting organism in one assay. The dot-blot assay seems to be more suitable for epidemiological studies, since large numbers of samples can be processed and screened at relatively low cost and with a limited amount of work. The in situ hybridization as described in this paper can be applied to cultures, smears and histological sections. Also, in situ hybridization can be performed within 3–4 h and without the use of radioisotopes. These properties make in situ hybridization suitable for investigation of clinical samples. Application of strain- or type-specific recombinant probes will add to the quality of the diagnosis. It might also provide tools for retrospective studies. We therefore think that the combination of specific DNA probes and the assays discussed may be a valuable addition in the diagnosis of leptospirosis in general and of hardjo infections in particular. This needs to be verified in field trials.

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