Detection of Leptospira interrogans in Clinical Specimens by in situ Hybridization Using Biotin-labelled DNA Probes

By W. J. TERPSTRA,¹* G. J. SCHOONE,¹ G. S. LIGTHART¹
AND J. TER SCHEGGET²

¹ Royal Tropical Institute, Department of Tropical Hygiene and WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis, Meibergdreef 39, 1105 AZ Amsterdam Z-O, The Netherlands
² University of Amsterdam, Department of Medical Microbiology, Amsterdam, The Netherlands

(Received 18 July 1986; revised 27 October 1986)

In situ DNA hybridization using biotin-labelled leptospiral DNA was performed on clinical specimens to investigate its usefulness as a technique for the identification of Leptospira interrogans. The applicability of this test in blood, urine and liver smears was demonstrated. In situ DNA hybridization can be completed in only 4 h and it combines the advantage of visualization of the leptospiral morphology with the specificity of the hybridization reaction. No cross-hybridization was observed with other bacteria. This study shows that hybridization in situ can be simple to perform and may contribute to a rapid diagnosis.

INTRODUCTION

DNA hybridization is rapidly gaining importance as a diagnostic tool in several infectious diseases (Meinkoth & Wahl, 1984), but the handling of radioactive probes and the elaborate extraction procedures used have been the main obstacles to widespread application of the technique. The use of the more stable biotin-labelled probes instead of radioactive probes makes the technique practicable in other than specially equipped laboratories (Langer et al., 1981). Biotin-labelled probes have been used successfully for the visual detection of DNA sequences specifically of viral origin in fixed eukaryotic cells with in situ hybridization (Brigati et al., 1983).

In this paper we describe our results with in situ hybridization on fixed whole leptospiral cells, using biotin-labelled DNA probes. We demonstrate the applicability of the technique in blood, urine and liver smears. Various other micro-organisms were tested to check for possible cross-hybridization which could interfere with the suitability of the test for clinical purposes. The test was also compared with immunoperoxidase staining (Terpstra et al., 1983).

METHODS

Preparation of samples for hybridization. (a) Various cultured bacteria. The following micro-organisms were examined for hybridization: Leptospira interrogans serovars copenhageni (strain Wijnberg) and hardjo (strain S, a cattle isolate), Leptospira biflexa serovar patoc (reference strain Patoc I), Borrelia burgdorferi, Campylobacter fetus subsp. jejuni, Vibrio cholerae, Yersinia enterocolitica, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus and Neisseria gonorrhoeae. The local strains Wijnberg and S and the saprophytic strain Patoc I are used routinely in the microscopic agglutination test for the serological diagnosis of human leptospirosis. The other bacteria were originally isolated from humans and are presently maintained in the laboratory for experimental purposes. For each species, a suspension was made in PBS (0.15 M-sodium chloride, 0.0067 M-sodium phosphate, pH 7.2) to contain approximately $10^7$ bacteria ml⁻¹. A drop of the suspension was placed on a microscope slide.

(b) Blood and liver smears. A virulent strain of serovar copenhageni was injected intraperitoneally in Syrian hamsters. After 3 d the animals became ill and were killed. Impression smears were made of their liver on microscope slides.
Blood was collected from a human patient in the first week of the disease. After two weeks leptospires were cultured from the blood which were subsequently typed as serovar copenhageni. From the same blood sample, plasma was centrifuged at 12000 g to concentrate leptospires. The sediment was suspended in saline and recentrifuged. The sediment was smeared on microscope slides.

(c) Urine. Urine was collected from a cow with naturally acquired leptospirosis. The urine was cultured in bovine albumin polysorbate medium (EMJH as described by Ellinghamus & McCullough (1965) and modified by Johnson & Harris (1967) (EMJH medium). The culture in EMJH medium enriched with 1% (v/v) rabbit serum (Ellis, 1986) yielded serovar hardjo. The urine was centrifuged for 15 min at 12000 g and the sediment smeared on to microscope slides.

All slides were dried and fixed for 10 min in methanol. Before hybridization, the slides were pretreated for 10 min with 1% (w/v) SDS in PBS containing 0.5 mg protease type 1 (Sigma) ml⁻¹, washed for 1 min in PBS, incubated for 10 min with 1% H₂O₂ (v/v) in PBS and rinsed in PBS.

Preparation of probe DNA. DNA was purified as described by Marshall et al. (1981), with a few modifications, from serovar copenhageni strain Wijnberg and serovar hardjo strain S. The leptospires were cultured in EMHJ medium and harvested by centrifugation. The sedimented cells were suspended in 10 mM-Tris/HCl buffer, pH 8.5, and 10 mM-sodium EDTA (TEB), washed twice in TEB and lysed by the addition of 1% (w/v) SDS. After incubation with pronase (1 mg ml⁻¹) for 18 h at 50 °C, NaCl was added to a final concentration of 1 M. The lysate was extracted three times with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). Nucleic acid was precipitated by ethanol and resuspended in 10 mM-Tris/HCl, 1 mM-sodium EDTA, pH 7.5 (TE) and treated with RNAase (100 µg ml⁻¹) at 37 °C for 30 min. DNA was extracted with the phenol/chloroform/isoamyl alcohol mixture, precipitated with ethanol, redissolved in TE and finally quantified spectrophotometrically. Total genomic DNA was labelled in vitro by nick translation with bio-11-dUTP according to the instructions of the manufacturer (Enzo Biochem) and as originally described by Langer et al. (1981) with the modification that the incubation period was extended to 6 h. In the copenhageni probe 42% and in the hardjo probe 20% of the available deoxythymidine residues in the DNA were substituted by biotin-labelled deoxyuridine. With a similarly prepared copenhageni probe it was possible to detect by dot blot hybridization 15 pg purified homologous DNA (Terpstra et al., 1986).

In situ hybridization. The hybridization solution contained 1-7 µg biotin-labelled probe DNA and 100 µg salmon sperm DNA ml⁻¹, in a mixture of 50% (v/v) formamide, 20% (w/v) dextran sulphate and 2 x saline sodium citrate (SSC: 0.15 M-NaCl and 15 mM-sodium citrate, pH 7). This hybridization mixture (20 µl) was dropped onto the sample preparations on glass slides; these were then covered with plastic tape to prevent evaporation.

The DNA on the slides was denatured by steam-heating for 10 min at 85 °C. Hybridization took place during incubation for 2 h at 33 °C in a humid chamber. After hybridization, the slides were washed for 10 min in 2 x SSC containing 50% formamide at 33 °C, for 10 min in 2 x SSC at room temperature and finally for 2 min in PBS. The slides were incubated for 30 min at 37 °C with a streptavidin-biotinylated-horseradish peroxidase complex in PBS containing 1% (w/v) BSA. The slides were washed for 1 min in PBS + 0.1% Triton X 100 and for 1 min in PBS.

The substrate was freshly prepared diaminobenzidine-tetrahydrochloride (DAB), 0.05% (w/v) in PBS with 0.01% H₂O₂; 100 µl of the substrate solution was dropped on each slide. The slides were then incubated in the dark for 10 min, and rinsed twice with distilled water. Peroxidase caused precipitation of the DAB, which resulted in brown stained leptospires. After drying, the slides were mounted in Aquamount and examined under the microscope (1000 x).

Immunoperoxidase staining. The same slides with blood, urine and liver smears used in the hybridization test were also processed for immunoperoxidase staining as described before (Terpstra et al., 1983). In brief, the fixed slides were incubated with rabbit anti-copenhageni or anti-hardjo serum, washed, incubated with peroxidase labelled sheep anti-rabbit serum, washed, incubated with substrate, 3-amino-9-ethylcarbazole (carbazole) or DAB, washed and mounted in glycerin (carbazole) or Aquamount (DAB).

RESULTS

To standardize the conditions for hybridization and to establish the potential usefulness of the DNA probes, the hybridization test was initially applied to various bacteria. Leptospires of serovar copenhageni and serovar hardjo of pathogenic L. interrogans were stained after hybridization with both the copenhageni and the hardjo probe, but the staining reaction was clearest when the homologous probe was used. Saprophytic L. biflexa, and all the other microorganisms tested, were not stained.

Leptospires in a liver smear (Fig. 1) of the hamsters infected with copenhageni were clearly visualized after hybridization with the copenhageni probe. In the plasma sediment (Fig. 2) of the human patient infected with copenhageni, leptospires were clearly visible using a copenhageni probe. After hybridization with the hardjo probe leptospires were visualized in the urine of the
DNA probes for leptospires

Fig. 1. *L. interrogans* serovar *copenhageni* in a liver smear of an experimentally infected golden hamster. (a) *In situ* hybridization using DAB as a substrate; (b) immunoperoxidase staining using carbazole as a substrate (bars, 10 μm).

Fig. 2. *L. interrogans* serovar *copenhageni* in the plasma sediment of a human patient. (a) *In situ* hybridization using DAB as a substrate. (b) Immunoperoxidase staining using carbazole as a substrate (bars, 10 μm).

Fig. 3. *L. interrogans* serovar *hardjo* in the urine of a cow. (a) *In situ* hybridization using DAB as a substrate; (b) immunoperoxidase staining using DAB as a substrate (bars, 10 μm).

cow infected with *hardjo* (Fig. 3). All observations were confirmed by immunoperoxidase staining.

**DISCUSSION**

In leptospirosis, conventional diagnostic methods, such as culturing, dark-field examination or silver staining, are either too slow or unreliable (Turner, 1970). DNA hybridization is a highly specific reaction (Meinkoth & Wahl, 1984). In this study we have demonstrated that *in situ* hybridization using biotin-labelled probes combines the specificity of the hybridization reaction...
with the visualization of the leptospiral morphology. After fixation and pretreatment of the samples to be examined, the cellular DNA is accessible to the biotinylated probe DNA. The whole bacterial cell is stained because DNA is evenly distributed throughout the cell. In situ DNA hybridization enabled the direct observation and identification of pathogenic leptospires in clinical samples of various origins.

Cross-hybridization was observed between serovars of pathogenic *L. interrogans*. The in situ hybridization reaction, as revealed by the intensity of the staining, was stronger with homologous than with heterologous DNA. The probes prepared of the pathogenic serovars caused no staining of leptospires of saprophytic *L. biflexa*. These observations are in accordance with our study on dot blot hybridization in which we showed that probes prepared from two serovars of *L. interrogans* with different G + C content cross-hybridized clearly but to varying degrees with DNA extracted from several other pathogenic serovars, while there was very little cross-hybridization with saprophytic *L. biflexa* (Terpstra et al., 1986). The varying degrees of cross-hybridization between pathogenic serovars was explained by differences in homology (Haapala et al., 1969; Brendle et al., 1974). From a clinical point of view it is important that the probe is species-specific and can be used to determine whether the patient has leptospirosis or not. The serovar-status of the leptospire causing the disease is of secondary importance. It is impractical to test for in situ cross-hybridization between all of more than 160 pathogenic serovars recognized so far but the results of this study suggest that probe DNA prepared from a single serovar can probably be used for the detection of all pathogenic serovars. Perhaps the use of a mixture of probe DNAs from different serovars might make the test more sensitive. The preparation of serovar-specific probes may be useful for epidemiological studies.

Morphologically the leptospires stained by in situ hybridization were quite similar to those stained with the immunoperoxidase method, but DNA hybridization gave much less background staining. The procedure described in this study was designed to minimize the time needed for hybridization. This was achieved by avoiding the time-consuming and elaborate extraction procedures necessary for filter hybridization and by keeping the hybridization time short. In situ DNA hybridization as described in this communication is a promising novel application of a well-established research technique in the rapid diagnosis of leptospirosis.

We want to express our sincere gratitude to Dr E. P. Wright for the critical reading of the manuscript.

**REFERENCES**


