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

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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 10⁷ 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 (EMHJ) as described by Ellinghausen & McCullough (1965) and modified by Johnson & Harris (1967) (EMHJ medium). The culture in EMHJ 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 RNase (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 with nick translation by using-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 sodium saline 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 ×).

Immunoperoxidase staining. The same slides with blood, urine and liver smears that were 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
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.

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REFERENCES


