Detection of leptospiral DNA by nucleic acid hybridisation with $^{32}$P- and biotin-labelled probes

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Summary. Dot hybridisation with $^{32}$P- and biotin-labelled probes prepared from leptospiral DNA was performed to develop a sensitive and specific diagnostic method for early infection with leptospires. The smallest amounts of leptospiral DNA that could be detected with $^{32}$P- and biotin-labelled probes was 1.5 pg and 5 pg, respectively, corresponding to about 750 and 2500 leptospires. Dot hybridisation with a $^{32}$P-labelled probe detected leptospiral DNA in sera from all of 14 experimentally infected golden hamsters. The smallest amount of leptospiral DNA detected in these experiments corresponded to about 2500 leptospires. In the test conditions described in this study, the sensitivity of dot hybridisation with a biotin-labelled probe was lower. Little cross-hybridisation was observed with unrelated DNAs which indicates that dot hybridisation could be a useful diagnostic method.

Introduction

Current methods for the detection of leptospires in clinical material are neither rapid nor reliable. Nucleic acid hybridisation with DNA probes has proved to be a rapid, sensitive and specific diagnostic technique in several infectious diseases (Meinkoth and Wahl, 1984) and could be useful in the early diagnosis of leptospirosis.

Earlier studies showed heterogeneity between DNAs of different serovars of pathogenic *Leptospira interrogans* (Haapala et al., 1969; Brendle et al., 1974; Marshall et al., 1981). In the light of these results it is questionable whether a DNA probe prepared from a single *Leptospira* strain could be used to detect different serovars. Therefore we studied the sensitivity and specificity of DNA hybridisation by the application of probes prepared from two strains, Wijnberg (serovar copenhageni) and Celledoni (serovar celledoni) with different guanine+cytosine (G+C) content (Haapala et al., 1969) on: (i) DNA extracted from serovars representing different serogroups of pathogenic leptospires; (ii) DNA from saprophytic leptospires; and (iii) DNA from unrelated sources.

DNA hybridisation is usually performed with radio-labelled probes. These require specially equipped laboratories which limits the applicability of hybridisation as a diagnostic method. The use of the more stable biotin-labelled DNA probes and the detection of labelled DNA hybrids by an enzymatic staining assay may be more practical for routine diagnosis (Leary et al., 1983). The aims of this study were to compare $^{32}$P-labelled probes with biotin-labelled probes and to test nucleic-acid hybridisation as a diagnostic method in experimentally infected golden hamsters.

Materials and methods

Purification of DNA

DNA was purified as described by Marshall et al. (1981) with the modifications that lysozyme treatment was omitted and that RNAase treatment was added to the procedure. Leptospires were cultured in bovine albumin polysorbate medium (Ellinghausen and McCullogh, 1965) as modified by Johnson and Harris (1967). This medium is referred to as EMJH medium. Leptospires in the culture were harvested by centrifugation at 12000 g for 30 min. The pellet was resuspended in 10 mM Tris-HCl buffer, pH 8.5, and 10 mM sodium EDTA (TEB). Cells were washed twice in TEB and lysed by 1% sodium dodecysulphate (SDS). The lysate was incubated with pronase 1 mg/ml for 18 h at 50°C. NaCl was added to a final concentration of 1 M. The lysate was extracted three times with a mixture of phenol, chloroform and iso-amylalcohol (25:24:1 v/v/v). The nucleic acid was precipitated by ethanol, resuspended in 10 mM Tris-HCl, I mM sodium EDTA, pH 7.5 (TE), digested with RNAase 100 µg/ml at 37°C for 30 min and again extracted with the phenol, chloroform, iso-amylalcohol mixture. DNA was
precipitated with ethanol, redissolved in TE and dialysed against TE.

This procedure was also used for the purification of DNA from Escherichia coli and Proteus mirabilis, except that, after harvesting, the cells were resuspended in TEB containing lysozyme 0.3 mg/ml. Human placental DNA was purified by essentially the same method except that before lysis the tissue was homogenised in a polytron (Kinematica GMBH, Switzerland).

DNA concentrations were determined spectrophotometrically.

In-vitro labelling of leptospiral DNA

DNA extracted from serovar copenhageni strain Wijnberg and serovar celledoni strain Celledoni was labelled in vitro by nick translation with \([\text{x-}^{32}\text{P}]\text{dTPP and}\) \([\text{x-}^{32}\text{P}]\text{dCTP} as described by Rigby et al. (1977). The specific activity of each probe was 1.5 \(\times\) 10^6 cpm/\(\mu\)g of DNA. Bio-1I-dUTP was incorporated according to the instructions of the manufacturer (Enzo Biochem, NY, USA) as originally described by Langer et al. (1981) with the following modifications: incorporation was monitored by the addition of trace amounts of \([\text{x-}^{32}\text{P}]\text{dCTP} and the incubation time was extended to 6 h. In DNA from serovars copenhageni and celledoni respectively, 31% and 39% of the available deoxythymidine residues were substituted by biotin-labelled deoxyuridine.

Nitrocellulose dotting

Serial dilutions were made of purified DNA in TE. Salmon sperm (5 \(\mu\)g/ml) DNA was added to the solution as a carrier. DNA was denatured by heating for 2 min at 100°C and 1 \(\mu\)l amounts of the DNA dilutions were dotted on a nitrocellulose membrane filter (Schleicher and Schüll, BA 85, W. Germany). The filter was dried in air and baked for 2 h at 80°C.

Filter hybridisation

All hybridisation and washing operations were performed as described by ter Schegget et al. (1980). The filters were pretreated for 10 min in 6 \(\times\) SSC and for 2 h at 68°C in a solution containing Ficoll 0.1%, polyvinylpyrrolidone 0.1% and bovine serum albumin (BSA) 0.01% in 6 \(\times\) SSC supplemented with low-molec.-wt denatured salmon-sperm DNA 100 \(\mu\)g/ml and SDS 0.5%. Hybridisation was performed for 18 h at 68°C in a solution containing salmon sperm DNA 100 \(\mu\)g, 1 mM EDTA and SDS 0.5% in 6 \(\times\) SSC, supplemented with dextran sulphate 20% to enhance the rate of hybridisation (Wetmur. 1975).

One \(\mu\)g of \(^{32}\text{P}-\text{or biotin-labelled leptospiral DNA was denatured at 100°C for 5 min and added to 30 ml or 6 ml of hybridisation solution respectively. After hybridisation the filters were washed at 68°C for several hours in 0.1 \(\times\) SSC containing SDS 0.5% before autoradiography, and in 2 \(\times\) SSC containing SDS 0.1% before the enzymatic staining method. For autoradiography, exposure was for 18 h. To demonstrate the hybridisation reaction with biotinylated probes the nitrocellulose filter was blocked with phosphate buffered saline (PBS), pH 7.2, containing BSA 2% and Triton X 100 0.1% for 30 min at 37°C. The filters were incubated with PBS containing streptavidin-biotinylated peroxidase complex and BSA 1% for 30 min at 37°C and then washed three times with a buffered salt solution, pH 6.5, containing 0.5 mM NaCl, 10 mM phosphate buffer, BSA 0.1%, and Tween 20 0.05%. Subsequently the filters were washed twice in a solution consisting of 2 \(\times\) SSC, BSA 0.1% and Tween 20 0.05%. Finally the filters were stained with tetramethylbenzidine (Merck, W. Germany) as described by Buckel and Zehelein (1981). Ten \(\mu\)l of melted agarose 0.7% (Litex LSA, Denmark) in 0.1 M citrate buffer adjusted to pH 5/0 with 1 M NaH_2PO_4 at 45°C was mixed with 8 mg of tetramethylbenzidine and 26.6 mg of diocetyl sodium sulphosuccinate (Merck) dissolved in 3.3 ml of ethanol. H_2O_2 was added to a final concentration of 0.01%. This solution was added to the filters for staining. Colour developed within 10 min after the addition of the substrate. This enzymatic staining procedure took 2 h.

Hamster experiment

Fourteen golden hamsters aged 6-8 weeks were given 0.5 ml of a culture of a virulent strain of L. interrogans serovar copenhageni by intraperitoneal injection. The leptospires were cultured for 4 days in EMJH medium. Three days after the inoculation the hamsters became ill and were killed. Their blood was examined for leptospires by dark-field microscopy and by culture in EMJH medium. For the purification of DNA, a rapid procedure (2 h) was devised. Volumes (0.5-1 ml) of serum were centrifuged in an Eppendorf centrifuge for 15 min at 8000 rpm to concentrate the leptospires in the sediment. To the sediment, 250 \(\mu\)l of SDS 1% in TE was added. Salmon sperm DNA (final concentration 5 \(\mu\)g/ml) was added as a carrier, followed by the addition of NaCl to a final concentration of 0.1 M. The lysate was extracted with a phenol, chloroform, iso-amyl alcohol mixture. DNA was precipitated by ethanol, denatured by the addition of 5 \(\mu\)l of 0.5 N NaOH and neutralised by adding 5 \(\mu\)l of a solution containing 0.5 \(\times\) HCl and 0.5 M Tris-HCl, pH 7.5. This is referred to as the serum extract.

From 10 \(\mu\)l of serum extract, serial tenfold dilutions were made in TE and 1 \(\mu\)l amounts of the dilutions were dotted on a nitrocellulose membrane filter. Sera from five uninfected hamsters were used as negative controls. As a positive control, leptospires of the non-virulent strain Wijnberg of serovar copenhageni cultured in EMJH medium were added to negative hamster serum to a final concentration of 1 \(\times\) 10^7 organisms/ml of serum. DNA was extracted from 25 \(\mu\)l of serum containing 25 \(\times\) 10^4 leptospires. From 10 \(\mu\)l of serum extract, 1 \(\mu\)l containing DNA corresponding to 25 \(\times\) 10^4 leptospires was dotted on the membrane filter. Probe DNA used in this experiment was prepared from the strain Wijnberg of serovar copenhageni.
Results

The amounts of DNA detected by the two probes are shown in the table. With radioactive and biotinylated probes prepared from two strains, considerable cross-hybridisation was observed between DNAs from pathogenic serovars. The detectable amounts of DNA were in the range 1.5–375 pg; 1 pg of leptospiral DNA was equivalent to c. 500 leptospires. This figure was estimated by measuring the amount of DNA extracted from cultures containing known numbers of leptospires. As expected, homologous DNA hybridisation permitted the detection of the smallest amounts of leptospiral DNA. The radioactive probes detected smaller amounts of DNA from pathogenic serovars than the biotinylated probes. For serovar copenhageni, the difference between the results obtained with the two types of probe was clearer than for serovar celledoni. Both types of probe cross-hybridised with DNA from saprophytic leptospires but only with relatively large quantities of these DNAs. Again the radioactive probes detected smaller quantities. The radioactive probes showed cross-hybridisation with DNA from Proteus mirabilis and also to a very slight extent with human DNA. With the biotinylated probes, no cross-hybridization was observed with DNAs from unrelated sources.

The fourteen hamsters were all infected with virulent leptospires as judged by positive results in dark-field examination and blood culture. The results of the hybridisation test are presented in the figure. Leptospiral DNA was detected with $^{32}$P- and biotin-labelled probes in all infected hamsters. In one hamster (lane 20) only a weak reaction was observed in the undiluted DNA extract of the serum. All other extracts gave positive reactions at dilutions of 1 in 10. Six serum extracts (lanes 11–19) gave positive results with the $^{32}$P-labelled probe at a dilution of 1 in 100 and one (lane 15) gave a positive result with the biotinylated probe at the same dilution. In the extract of the positive control serum (lane 6) hybridisation was observed at a dilution of 1 in 10 with a $^{32}$P-labelled probe. This corresponded to approximately 2500 leptospires. Using the biotin-labelled probe, hybridisation was observed only in the undiluted positive control serum, corresponding to approximately 25 000 leptospires. No hybridisation reactions were observed with the extracts of the negative control sera (lanes 1–5).

Table. The smallest quantities of DNA from serovars of pathogenic L. interrogans (1–10), saprophytic L. biflexa (11–12) and various other sources (13–15) detected with $^{32}$P- and biotin-labelled probes prepared from serovars copenhageni strain Wijnberg and celledoni strain Celledoni.

<table>
<thead>
<tr>
<th>Hybridisation with DNA from</th>
<th>Mean values of smallest quantities (pg) of DNA detected on duplicate filters with DNA probe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{32}$P</td>
</tr>
<tr>
<td>1. serovar copenhageni</td>
<td>3</td>
</tr>
<tr>
<td>2. serovar hardjo</td>
<td>15</td>
</tr>
<tr>
<td>3. serovar pomona</td>
<td>3</td>
</tr>
<tr>
<td>4. serovar bataviae</td>
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<tr>
<td>5. serovar bratislava</td>
<td>13</td>
</tr>
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<td>6. serovar hebdomadis</td>
<td>1-5</td>
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<tr>
<td>7. serovar poi</td>
<td>40</td>
</tr>
<tr>
<td>8. serovar celledoni</td>
<td>20</td>
</tr>
<tr>
<td>9. serovar tarassovi</td>
<td>20</td>
</tr>
<tr>
<td>10. serovar ranarum</td>
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</tr>
<tr>
<td>11. serovar andamana</td>
<td>$3.8 \times 10^3$</td>
</tr>
<tr>
<td>12. serovar patoc</td>
<td>$2.5 \times 10^3$</td>
</tr>
<tr>
<td>13. Proteus mirabilis</td>
<td>$3.8 \times 10^3$</td>
</tr>
<tr>
<td>14. Escherichia coli</td>
<td>negative*</td>
</tr>
<tr>
<td>15. human placenta</td>
<td>$6 \times 10^3$</td>
</tr>
</tbody>
</table>

* Negative = no signal with $2 \times 10^4$ pg of DNA.
† Negative = no signal with $1 \times 10^6$ pg of DNA.
Figure. Dot hybridisation with a $^{32}$P-labelled probe (A) or a biotin-labelled probe (B) on undiluted and tenfold dilutions of extracts of sera from uninfected hamsters (lanes 1–5), a positive control serum (lane 6) and sera from experimentally infected hamsters (lanes 7–20). Control DNA was DNA purified from serovar *copenhageni*. 
Discussion

The smallest amount of purified leptospiral DNA that could be detected by dot hybridisation corresponded to c. 750 leptospires. This is comparable with the results of a similar study on *Legionella* which detected 400 micro-organisms (Köhne et al., 1984). However, the minimum of DNA we detected varied considerably between serovars. This variation can probably be attributed to differences in homology between lepospiral DNAs. Although Haapala et al. (1969) and Brendle et al. (1974) used a different hybridisation procedure, the finding by these workers of genetically distinct groups among pathogenic leptospires may explain the variable degrees of hybridisation observed in our study.

In the test conditions described in this study, the radioactive probes were generally more sensitive than the biotinylated probes. When the results are compared it should be realized that, after hybridisation with the $^{32}P$-labelled probes, the filters were washed under more stringent conditions than after hybridisation with the biotin-labelled probes. It is important for an optimal signal-to-noise ratio that the hybridising DNA is able to form “networks” or “hyperpolymers” (Meinkoth and Wahl, 1984). Langer et al. (1981) stated that DNA in which every TMP residue in one strand is replaced by bio-dUMP residues has a melting temperature which is 5°C lower than that of the unsubstituted DNA. Although the effect of this substitution in the experiments described by Langer et al. is fairly small, it could be of greater significance on the melting temperature of “hyperpolymers”. In that case stringent washing conditions may lead to a low signal-to-noise ratio. For this reason we used less stringent washing conditions after hybridisation with the biotinlabelled probes. In spite of the low stringency with these probes, a similar specificity was obtained to that with the $^{32}P$-labelled probes, but reducing the stringency of the washing conditions after hybridisation with the $^{32}P$-labelled probes will probably lead to a higher sensitivity at the cost of the specificity.

Dot hybridisation with both types of probe detected leptospiral DNA in the serum of all experimentally infected hamsters, but, as in the in-vitro work, dot hybridisation with $^{32}P$-labelled probes was generally the most sensitive method. These observations indicate that the radioactive probes had a high degree of sensitivity and a sufficient degree of specificity to be useful for the detection of leptospires in clinical material. Non-radioactive probes would be more valuable as a diagnostic tool, because they do not require a specialised laboratory, give quicker results and can be stored for a longer time. Dot hybridisation with biotin-labelled leptospiral DNA had a good degree of specificity and a fair degree of sensitivity, and can probably fulfil the requirements for a useful diagnostic tool. The sensitivity of the non-radioactive probe might be further increased, e.g., by the application of complexes of avidin with enzyme polymer (Leary et al., 1983). The sensitivity of the hybridisation test can also be increased by centrifuging and extracting larger amounts of serum and by spotting larger quantities of the extract on the nitrocellulose membrane filters.

Further studies are needed to confirm that contaminating DNA from unrelated sources, such as leukocytes or bacteria in clinical material, will not interfere with the test.

Although considerable cross-hybridisation was observed among pathogenic leptospires, up to 100-fold differences were observed between the lowest detectable quantities of DNA derived from different serovars. This means that the use of a probe prepared from a single strain may be insufficiently sensitive to detect distantly related pathogenic serovars. It may be possible to prepare a DNA probe by molecular cloning which is able to detect most pathogenic serovars with about the same sensitivity, or alternatively to use a mixture of probes prepared from different serovars.

REFERENCES


