DNA PROBES FOR IDENTIFICATION OF LEPTOSPIRES AND DISEASE DIAGNOSIS

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Abstract. A newly identified 1 kb DNA fragment amplified by PCR using $(AG)_8T$ inter-simple sequence repeats (ISSR) primer and a 631bp segment of 16S rRNA ribosomal gene amplified by PCR using reported primers were labeled with a $\alpha^{32}P$ dCTP for use as DNA probes. These probes were hybridized with DNA extracted from 19 standard pathogenic serovars, 3 standard saprophytic serovars, 33 pathogenic isolates (12 from patients, 1 from a tapwater source, and 20 from rodents), and 22 saprophytic isolates from environmental sources. The pathogen-specific 16S rRNA DNA probe specifically hybridized all 33 standard pathogenic serovars, to 13 pathogenic isolates. Similarly, the saprophyte specific 1kb ISSR DNA probe specifically hybridized the 3 standard saprophytic serovars and the 22 saprophytic *Leptospira* isolates. The sensitivity of the 1 kb labeled saprophytic *Leptospira* specific DNA probe was 1.95 ng, and for the 16S rRNA pathogen specific probe 3.90 ng. The 16S rRNA gene segment DNA probe could also identify the leptospiremic stage in mice or guinea pigs infected experimentally with the pathogenic serovars *australis*, *autumnalis* or *icterohaemorrhagiae*. DNA probes therefore, owing to their high specificity and sensitivity, appear useful for easy, rapid, and reliable differentiation of pathogenic *Leptospira* strains and also hold promise for direct identification of organisms in blood samples to diagnose leptopsirosis.

INTRODUCTION

Isolation of causative *Leptospira* serovars from clinical samples provides confirmatory diagnosis, but may take 2 to 4 weeks and, therefore, does not aid patient management. Moreover, there are more than 200 *Leptospira* serovars with pathogenic potential and over 60 serovars identified as non-pathogens that are indistinguishable morphologically or by growth characteristics. During processing of clinical samples for isolation of the organisms there is a possibility of contamination by saprophytic strains, which may create confusion. Differentiation of pathogenic leptospires from non-pathogenic ones also assumes importance, as the presence of pathogenic leptospires in lakes and streams is an index of leptospirosis in wildlife or domestic animals having access to these waters (Kelley, 1992).

Molecular methods have recently been attempted as an alternative to culture and for broad group differentiation of an isolate to achieve rapid and reliable results. Several primer pairs for PCR detection of leptospires have been described, some based on specific gene targets, most frequently 16S or 23S rRNA genes, and repetitive elements, while others have been constructed from genome libraries. However, few have been shown to amplify leptospiral DNA from either human or veterinary clinical material and of these, only 2 methods have been subjected to extensive clinical evaluation. Both these methods have limitations. The primers described by Merien *et al*

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(1992), amplify a 331 bp fragment of the 16S rRNA gene of both pathogenic and saprophytic leptospires, whereas the G1 and G2 primers described by Gravekamp *et al* (1991) do not amplify all the pathogenic serovars.

DNA probes for use in clinical material have been attempted but were restricted to specific serovar detection *eg* serovar *hardjo-bovis* for detection of leptospires in bovine urine (LeFebvre, 1987). A fluorogenic DNA probe has also been reported for specific hybridization of pathogenic serovars at the 23S rRNA gene fragment (Woo *et al*, 1997a). In the present study, PCR amplified ISSR fragment specific for saprophytic strains (Shukla *et al*, 2001) and the pathogenic serovar specific 631bp PCR amplified segment of the 16S rRNA gene were labeled with 32P dCTP used as probes to differentiate strains. The pathogencic serovar-specific probe was also used to detect leptospiremic stages in experimentally infected mice and guinea pigs.

MATERIALS AND METHODS

Leptospira serovars and isolates

Pathogenic serovars of *Leptospira* species, viz, *L. interrogans* serovars *bataviae, australis, autumnalis, canicola, copenhageni, hebdomadis, icterohaemorrhagiae* and *djasmin; L. kirschneri* serovars *cynopteri* and *grippotyphosa; L. santarosai* serovars *shermani* and *celledoni*; *L. weilii* serovar *sarmin* and *L. borgpetersenii* serovars *ballum, javanica* and *tarassovi*, and the non-pathogenic *Leptospira* species *L. biflexa* serovar *patoc* and *L. meyeri* serovar *ranarum* were obtained from the WHO Collaborating Center, Netherlands. Pathogenic serovars of *Leptospira* species *L. interrogans* serovar *pomona* and *hardjo*; *L. weilii* serovar *manhao*; and the non-pathogenic *Leptospira* species *L. biflexa* serovar *andamana* were obtained from the National Leptospirosis Reference Center, Regional Medical Research Center (ICMR), Port Blair India. These serovars were maintained in Ellinghausen McCollough, Johnson and Harris (1967) (EMJH, Difco, USA) media for use in the present work. Twelve isolates of *Leptospira* recovered from hospitalized patients with pyrexia of unknown origin (PUO) and/or febrile jaundice, 23 isolates obtained from different water sources (sewage, stagnant water, and tap water) and 20 isolates recovered from trapped wild rodents from Gwalior City, were also utilized in testing DNA probes. The *Leptospira* isolates were identified as pathogenic or saprophytic on the basis of the conventional 8-azaguanine test (Johnson and Rogers, 1964), PPD dye test (Fuzi and Csoka, 1961b), egg-yolk reaction test (Fuzi and Csoka, 1961a) and growth at 13ºC (Johnson and Harris, 1967), and also by PCR using reported 16S rRNA and 23S rRNA differentiating primers (Hookey, 1992; Woo *et al*, 1997 b).

DNA extraction

Leptospira cultures at 10-15 days' growth were harvested by centrifugation at 12,000*g* for 30 minutes at 4ºC. The pellet was suspended in GTE buffer (50 mM glucose, 1M tris-HCl pH 8.0, 0.5 M EDTA pH 8.0) and then resuspended in lysis buffer (10% SDS and 10 N NaOH). This was then subjected to phenol-chloroform extraction twice, and precipitated with ethanol. After two washes in 70% ethanol, the DNA preparation obtained was air-dried and re-dissolved in ultra-pure autoclaved water (Brendle *et al*, 1974).

Preparation of DNA probes

The 1 kb fragment obtained from saprophytic species *L. biflexa* seovar *patoc* following PCR amplification with $(AG)_8T$ primer was used for preparation of the probe. Similarly, the 631 bp region of the 16S rRNA gene specific for pathogenic species of *Leptospira* was labeled following its amplification from *L. interrogans* serovar *australis*. Labeling of DNAs was carried out as described by Rigby *et al* (1977), using α^{32}P dCTP in nick translation kit obtained from Amersham Pharmacia Biotech, UK.

Dot-blot hybridization

DNA samples from standard serovars and isolates were spotted onto 2 sets of nylon membrane. One set was utilized for 1 kb labeled probe and the second set was used for 631 bp labeled probe. Hybridization was performed as per the method of Wetmur (1975). Briefly, the spotted DNA samples on nylon membrane were denatured in 2N NaOH for 15 minutes and then neutralized in 2M tris-Cl (pH 7.5) for 15 minutes followed by washing in 1x SSC buffer for 15 min-

utes. After drying in air and baking at 80°C for 2 hours, the membrane was subjected to pre-hybridization in 10 ml solution containing dextran sulfate (1g), formamide (5 ml), SDS (100 mg) and NaCl (0.58 g) supplemented with denatured salmon sperm DNA (10 μg) for 4 hours at 42°C. Hybridization was carried out in 10 ml of solution containing 1 mM EDTA, 5% SDS and 100 μl reaction mixture of labeled probe for 18 hours at 42°C.

After hybridization, the filters were washed for 15 minutes with 1xSSC containing 10% SDS at room temperature, and then for 15 minutes at 50°C with 20x SSC solution containing 10% SDS. Autoradiography was performed at -70°C for 18 hours on Kodak x-ray film.

Sensitivity of the probes

The sensitivity of the 1 kb ISSR probe was tested by spotting different concentrations of DNA extracted from *L. biflexa* serovar *patoc*, ranging from to 1 μg to 0.0305 ng, onto nylon membrane. Similarly, the sensitivity of the 16S rRNA probe was tested on DNA extracted from *L. interrogans* serovar *australis*. The hybridization procedure followed was the same as mentioned above.

Experimental infection in mice:

Swiss albino mice, 5 in each group, weighing 15-20 g were inoculated intraperitoneally with 1 ml of 7-10-day-old culture of either serovar *australis* or *icterohaemorrhagiae* grown in EMJH medium. The mice from each group were bled by ocular puncture on 0,2,4,6,8 and 10 days post-inoculation (DPI), and approximately 2 ml of pooled blood was collected in heparinized vials for each group. A drop of this pooled blood was added to 5 ml of EMJH medium supplemented with 200 μg/ml of 5-fluorouracil for culture and further 10-fold dilutions were also made. The tubes were incubated at 28°C for 45 days. Plasma was centrifuged at 3,000 rpm for 10 minutes to sediment the RBC, followed by 10,000 rpm for 30 minutes to sediment the organisms. The supernatant was discarded and the sediment was re-suspended in 75 μl of sterile PBS. This sample preparation was used for dark field microscopy (DFM), PCR and DNA probe.

Experimental infection in guinea pigs

A group of 6 guinea pigs weighing 400-600 g was inoculated with 0.5 ml of 10-day-old culture of serovar *autumnalis* by intraperitoneal route. The blood was collected by cardiac puncture at 0, 2, 4, 6, 8 and 10 DPI from individual animals and processed for DFM, culture, PCR, and DNA probe.

Dark field microscopy

Ten μl of the plasma from 0, 2, 4, 6, 8, and 10 DPI were placed on a thin microscopic glass-slide and a 22-mm^2 coverslip was placed over it to make a thin film. It was then examined under dark field microscope at a magnification of 40x.

Polymerase chain reaction

The pellet obtained following centrifugation of plasma from blood at different DPI was re-suspended in 50 μl of sterile PBS and processed for DNA preparation using QIAGEN Tissue kit. PCR amplification was performed with pathogenic serovar specific primers of the 16S rRNA gene, using the following steps: denaturation at 94ºC for 1 minute, annealing at 50ºC for 1 minute and extension at 72ºC for 2 minutes, for 35 cycles, followed by 10 minutes extension at 72ºC. Each 25 ml PCR reaction contained 2.5 mM $MgCl_2$, 200 μM dNTPs, 50 mM KCl, 10 mM tris-HCl, 1% Triton X-100, 1 unit of *Taq* DNA polymerase, 5 p moles of primers, and 30 ng of genomic DNA. The amplified products were detected by 0.8% agarose gel electophoresis.

DNA probe

The pellet obtained following centrifugation of plasma from blood at different DPI was re-suspended in 50 μl of sterile PBS and processed for DNA preparation using QIAGEN Tissue Kit and processed for DNA hybridization. The protocol followed for the DNA probe was as mentioned earlier.

RESULTS

All the 12 isolates from PUO/febrile jaundice patients, 20 rodent isolates, and 1 isolate from a tapwater source, were identified as pathogenic, while the remaining 22 isolates from different water sources (sewage, stagnant water, and tap water) were saprophytic by the conventional tests, 8-azaguanine, PPD dye, egg-yolk reaction and

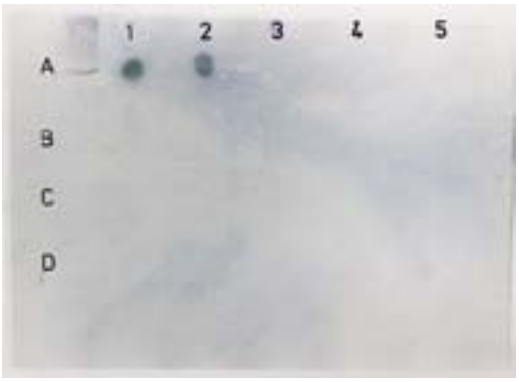

Fig 1– Dot-blot hybridization with standard *Leptospira* serovars using 1 kb ISSR region probe. Lanes : A1- *patoc,* A2- *ranarum,* A3- *australis,* A4- *autumnalis,* A5- *bataviae;* B1- *canicola,* B2- *hebdomadis,* B3- *icterohaemorrhagiae,* B4- *icterohaemorrhagiae copenhagnii,* B5- *djasmin;* C1- *cyanopteri,* C2- *grippotyphosa,* C3- *shermani,* C4- *celledoni* C5- *sarmin;* D1- *ballum,* D2- *javanica,* D3- *tarassovi.*

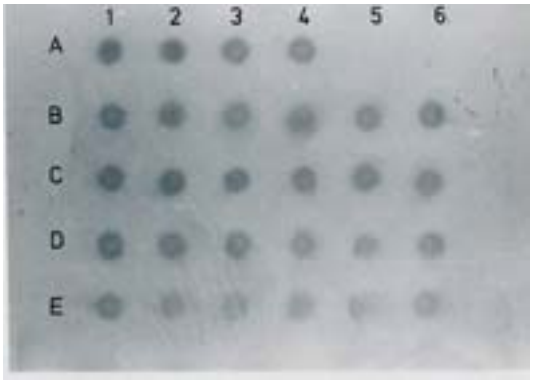

Fig 2–Dot-blot hybridization with standard *Leptospira* serovars and pathogenic isolates using 631 bp 16S rRNA probe. Lanes : A1- *australis,* A2- *autumnalis,* A3- *canicola,* A4- *bataviae,* A5- *patoc,* A6- *ranarum*; B1- *hebdomadis,* B2- *icterohaemorrhagiae*; B3- *icterohaemorrhagiae copenhagnii,* B4- *djasmin,* B5- *cyanopteri,* B6- *grippotyphosa,* C1- *shermani,* C2- *celledoni,* C3- *sarmin,* C4- *ballum,* C5- *javanica,* C6- *tarassovi*; D1-D6 and E1-E6 - all pathogenic isolates recovered from human patients.

growth at 13ºC, and also by PCR using the reported 16S rRNA and 23S rRNA differentiating primers.

The 1 kb ISSR probe hybridized to the DNAs extracted from standard saprophytic *Leptospira* species, namely, *Leptospira biflexa* serovar *patoc, andamana* and *L. meyeri* serovar *ranarum*. No spot was observed with DNAs extracted from 19 standard pathogenic serovars tested, namely, *Leptospira interrogans* serovars *bataviae, australis, autumnalis, canicola, copenhageni, hebdomadis, icterohaemorrhagiae, icterohaemorrhagiae copenhagnii, djasmin; pomona; hardjo*; *Leptospira kirschneri* serovars *cyanopteri, grippotyphosa; Leptospira santarosai* serovars *shermani, celledoni*; *Leptospira weilii* serovar *manhao, sarmin;* and *Leptospira borgpetersenii* serovars *ballum, javanica* and *tarassovi* (Fig 1).

The 631 bp 16S rRNA probe revealed hybridization to the DNAs extracted from all the 19 standard pathogenic *Leptospira* serovars tested and not to the DNAs from *L. biflexa* serovar *patoc, andamana* and *L. meyeri* serovar *ranarum* (Fig 2).

After testing the probes on the standard strains of *Leptospira* species, these were then evaluated on *Leptospira* isolates. DNA from all the 33 isolates identified as pathogenic by conventional tests and PCR, hybridized with the pathogen specific 631bp fragment of the 16S rRNA probe, and not with the 1 kb ISSR probe. Conversely, DNA from the 22 saprophytic isolates hybridized only to the 1 kb ISSR probe and not to the pathogen specific 631bp of the 16S rRNA probe (Figs 3a, 3b, 4, 5).

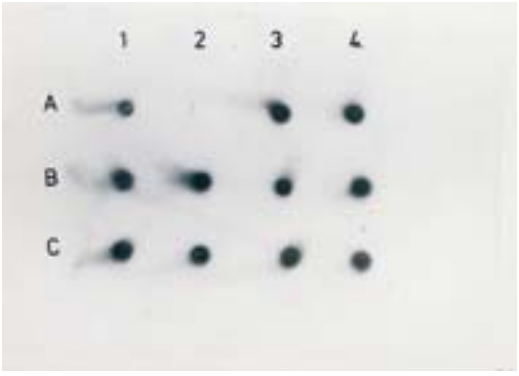

Fig 3a–Dot-blot hybridization with *Leptospira* isolates recovered from rodents using 631 bp 16S rRNA probe. Lanes : A1- *australis,* A2- *patoc,* A3, A4, B1- B4 and C1- C4 – all pathogenic isolates from rodents.

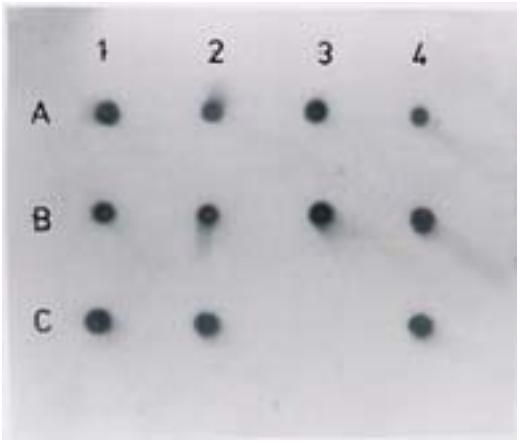

Fig 3b–Dot-blot hybridization with *Leptospira* isolates recovered from rodents using 631 bp 16S rRNA probe. Lanes : A1- A4, B1- B4, C1 and C2 -rodents isolates, C3- *patoc*, C4- *autumnalis*.

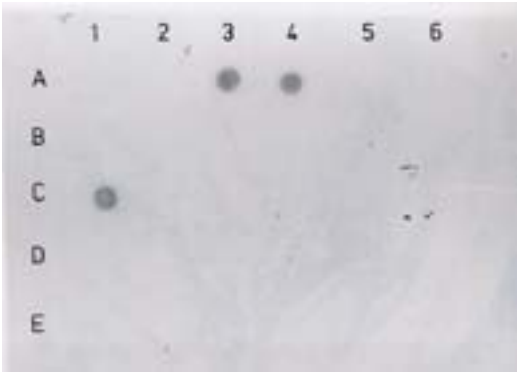

Fig 4–Dot-blot hybridization with *Leptospira* isolates recovered from water sources using 631 bp 16S rRNA probe. Lanes : A1- *patoc*, A2- *ranarum*, A3- *grippotyphosa*, A4- *hebdomedis*, A5, A6 and B1- B6 -saprophytic isolates, C1-pathogenic isolate recovered from tap water source. C2- C6, D1- D6 and E1 – E3 - saprophytic isolates.

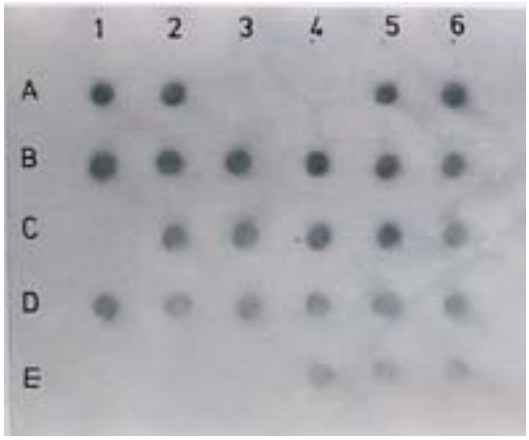

Fig 5–Dot-blot hybridization with *Leptospira* isolates recovered from water sources using 1 kb ISSR region probe. Lanes : A1-*patoc*, A2-*ranarum*, A3-*ballum*, A4-*bataviae*, A5, A6 and B1-B6 - saprophytic isolates; C1-tap water pathogenic isolate, C2-C6, D1-D6 and E4- E6 saprophytic isolates, E1-E3-blanks.

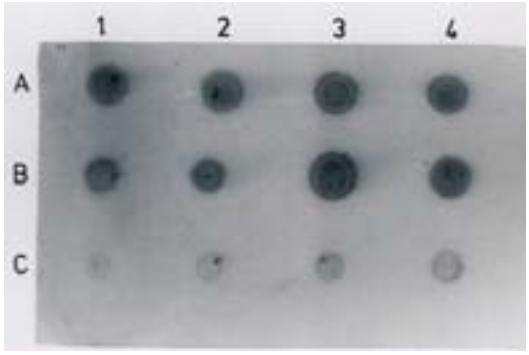

Fig 6–Sensitivity of 1kb saprophytic ISSR DNA probe. Lanes : A1- 1,000 ng, A2- 500 ng, A3- 250 ng, A4- 125 ng, B1- 62.5 ng, B2- 31.25 ng, B3- 15.62 ng, B4- 7.81 ng, C1- 3.90 ng, and C2- 1.95 ng.

The sensitivity of the 1 kb ISSR region probe was found to be 1.95 ng when tested on the DNA from *L. biflexa* serovar *patoc* (Fig 6) and that of the 631 bp 16S rRNA probe was 3.90 ng on the DNA extracted from *L. interrogans* serovar *australis* (Fig 7).

Experimental infection in mice

Demonstration of leptospires in mice experimentally infected with serovar *australis* by DFM and PCR and by pathogenic-specific 631 bp of the 16S rRNA probe, could be observed only on the 2nd DPI. The leptospiremic stage could be detected by PCR and DNA probe up to the 8th day, by culture up to the 6th day, and by DFM up to the 4th day of testing (Table 1).

In mice infected with serovar *autumnalis*, the organisms could be seen in DFM till 4 DPI, by culture, PCR, and DNA probe up to 6 DPI (Table 2).

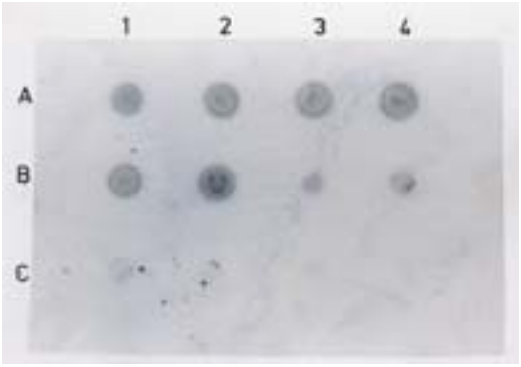

Fig 7–Sensitivity of 631bp 16S rRNA pathogenic specific probe. Lanes : A1- 1,000 ng, A2- 500 ng, A3- 250 ng, A4- 125 ng; B1- 62.5 ng, B2- 31.25 ng, B3- 15.62 ng, B4- 7.81 ng; C1- 3.90 ng.

Experimental infection in guinea pigs

In all guinea pigs, by DFM, the organisms could be visualized till 2 DPI, in 4 guinea pigs till 4 DPI, in 3 till 6 DPI, and in 2 till 8 DPI. No organisms were seen at 10 DPI. In PCR, amplification could be obtained in all 6 guinea pigs till 6 DPI, and like wise, DNA probe also detected the presence of the organisms till 6 DPI. The organisms could be isolated from all 6 guinea pigs till 4 DPI, from 3 guinea pigs till 6 DPI, and from 1 till 8 DPI (Table 3).

DISCUSSION

A rapid, simple and convenient differentiation method for identifying an isolate or a strain into broader groups of pathogenic or saprophytic leptospires is helpful both to the clinician and the epidemiologist. The conventional methods of differentiation, *ie*, 8-azaguanine test, PPD dye test, egg-yolk reaction test and growth at 13ºC, though reliable, may take up to 2-4 weeks. In recent years, PCR methods have become available for rapid differentiation of pathogenic and saprophytic serovars of *Leptospira* based on conserved regions located in rRNA genes of both 16S and 23S ribosomes, and also on repetitive elements, like the newly-identified ISSR region (Shukla *et al*, 2001). A fluorescent probe derived from the PCR-amplified region of 23S rRNA, which could successfully identify 23 strains of 6 pathogenic *Leptospira* genospecies and 8 strains of saprophytic *Leptospira biflexa*, has been reported. The probes described in the present study, making use of PCR-amplified 32P dCTP-labeled 631bp fragment of 16S rRNA gene and the 1 kb ISSR region, similarly provide clear specificities both for pathogenic and saprophytic strains when tested on standard serovars and isolates from different sources, and, therefore, can be of help in identifying isolates into broader classifications of pathogens or non-pathogens.

Table 1
Experimental infection in mice with serovar *australis.*

DPI	DFM	Culture	PCR	DNA probe
0	-	-	-	-
2	+	+	+	+
4	+	+	+	+
6	-	+	+	+
8	-	-	+	+
10	-	-	-	-

Table 2
Experimental infection in mice with serovar *autumnalis.*

DPI	DFM	Culture	PCR	DNA probe
0	-	-	-	-
2	+	+	+	+
4	+	+	+	+
6	-	+	+	+
8	-	-	-	-
10	-	-	-	-

Table 3
Experimental infection in guinea pigs with serovar *icterohaemorrhagiae.*

DPI	Animals tested	Animals positive			
		DFM	Culture	PCR	DNA probe
0	6	0	0	0	0
2	6	6	6	6	6
4	6	4	6	6	6
6	6	3	3	6	6
8	6	2	1	0	0
10	6	0	0	0	0

PCRs have been attempted to achieve rapid and reliable results for identifying *Leptospira* infection, but were mostly based on the use of primers that failed to differentiate pathogenic and saprophytic strains, with the associated limitation of false positive results in case of contamination by saprophytic leptospires at the time of sample processing (Levett, 2001). Reproducibility, and testing on large numbers of samples are other limitations before being acceptable as reliable tests. Moreover, PCRs are not yet available to identify infecting serovars. This may not be a significant limitation for the clinician managing a patient. The earliest availability to confirm the identity of an isolate into a broader pathogenic or non-pathogenic group is sufficient to initiate specific therapy, which fortunately remains common for all infecting serovars. The 16S rRNA probe described here, with sensitivity comparable to culture and PCR during the leptospiremic stage in experimentally-infected mice or guinea pigs, with any of the 3 *Leptospira* serovars *australis*, *autumnalis* and *icterrohaemorrhagiae* tested, may have ultimate utility for disease diagnosis. This DNA probe has the potential of being adapted to enzyme-based cold probe for possible application in routine microbiology laboratory. In a preliminary study undertaken on 5 culture proven cases, the hybridization with 631 bp 16S rRNA pathogenic serovar specific DNA probe could be observed with DNA extracted from plasma samples of all 5 patients. Once evaluated with large numbers and found satisfactory, this DNA probe may have merits over existing leptospirosis diagnostic systems.

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