Inter-simple-sequence repeat (ISSR)-PCR for the identification of saprophytic strains of Leptospira

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Summary

The inter-simple-sequence repeat (ISSR) primers that anneal to a simple repeat of various length and at non-repetitive motifs at 3’ and 5’ end were attempted for PCR amplification of Leptospira genome. Of the six ISSR primers tested, namely, (AG)₆T, (AG)₆C, (AG)₆G, (CA)₆A, (TG)₆C and (TG)₆G, only primer (AG)₆T produced amplification of 1000 bp in the two non-pathogenic Leptospira species tested, viz: Leptospira biflexa serovar patoc and L. meyeri serovar ranarum, with no amplification in any of the 16 standard pathogenic serovars tested. The remaining five ISSR primers did not exhibit any amplification of the Leptospira genome in either pathogenic or non-pathogenic species. From among 35 Leptospira isolates recovered from hospitalized patients with pyrexia of unknown origin and/or febrile jaundice (12 in number) and from different environmental water sources (23 in number), (AG)₆T ISSR-PCR correctly identified all the 22 isolates from water sources that were confirmed to be non-pathogenic by conventional tests. The results therefore, confirmed the ability of a primer, based on simple-sequence repeat motif, to produce a fragment that is useful as a group genetic marker in Leptospira species. The single nucleotide anchor, T, at the 3’ end of the primer appeared to play an important role in differentiation of pathogenic and non-pathogenic species of Leptospira. Multiplex PCR, using ISSR primer, (AG)₆T and the reported 16S rRNA gene primers, specific for pathogenic Leptospira species, or the 23S rRNA Leptospira genus specific primers, provided clear identification of serovars and isolates into pathogenic or non-pathogenic groups.

Introduction

Leptospirosis is one of the world’s most important zoonotic diseases. It is primarily an infectious disease of ruminants like cattle, sheep and shrews etc. Man usually contracts the disease by coming into contact with infected animals or contaminated animal products. Leptospira organisms can be found virtually in all tropical and temperate areas of the world and a large number of serovars distinguishable into broad categories of pathogenic and non-pathogenic groups have been reported. These are indistinguishable under dark field microscopy and by culture characteristics. Inhibitory or support growth in 8-azaguanine-containing media (Johnson & Rogers 1964), egg-yolk reaction test (Fuji & Csoka 1961a), p-phenylenediamine (PPD) dye test (Fuji & Csoka 1961b) and growth at 13 °C (Johnson & Harris 1967) are the conventional methods of differentiation into pathogenic and non-pathogenic groups and may take up to 2-4 weeks to obtain the results. PCR amplification of the specific 16S rRNA region (Hookey 1992) and of the 23S rRNA region (Woo et al. 1997a) have been recently reported for differentiation.

Inter-simple-sequence repeats (ISSRs) are DNA markers that involve the use of microsatellite sequences directly in the PCR for the DNA amplification (Zietkiewicz et al. 1994). Microsatellite or simple-sequence repeat (SSR) are short tandem repetitive DNA sequence with a repeat length of 1–5 bp (Litt & Luty 1989). Multilocus fingerprinting methods have been developed using oligonucleotides based on SSRs as primers in PCR amplification (Gupta et al. 1994; Sanchez et al. 1996). These methods enable amplification of genomic DNA and provide information about many loci simultaneously. These markers have been used for genetic diversity, gene tagging and genome mapping in plant species (Tsunura et al. 1996; Ratnaparkhe et al. 1998a, b). Microsatellites have been characterized in number of microorganisms (Van Belkum et al. 1998), however, ISSR have not been used for the study of microorganisms of clinical importance. We report here an ISSR-PCR that appears to differentiate pathogenic and non-pathogenic Leptospira serovars.
Materials and methods

Leptospira serovars

Pathogenic serovars of *Leptospira* species, viz., *Leptospira interrogans* serovars bataviae, australis, canicola, copenhageni, hebdomadis, icterohaemorrhagiae and djasmin, *Leptospira kirschneri* serovars cynopteri and grippotyphosa; *Leptospira santarosai* serovars shermani and celledoni; *Leptospira weilii* serovar sarmin and *Leptospira burgdorferi* serovars hallum, javanica and tarassovi and the saprophytic *Leptospira* of species *Leptospira biflexa* serovar patoc and *Leptospira meyeri* serovar ranarum were obtained from WHO Collaborating Center, Netherlands. These serovars were maintained in EMJH media. Twelve isolates of *Leptospira* recovered from hospitalized patients of pyrexia of unknown origin (PUO) and/or from cases of febrile jaundice and also 23 isolates obtained from different water sources of sewage, stagnant water and tap water of Gwalior city were utilized in the present study.

DNA extraction

For extraction of DNA, exponentially growing leptospire cultures were centrifuged at 12,000 × g for 30 min at 4 °C. The pellet was suspended in glucose–tris–EDTA buffer (50 mM Glucose, 1 M Tris–HCl, 0.5 M EDTA, pH 8.0) and then resuspended in lysis buffer (10% SDS and 10 NaOH). This was then subjected to phenol–chloroform extraction twice and precipitated with ethanol. After two washes in 70% ethanol, the DNA preparation thus obtained was air dried and redissolved in ultrapure water (Brendle et al. 1974).

PCR primers

The set of ISSR primers were obtained from University of British Columbia, Vancouver, Canada. The primers utilized were of 17 nucleotides in length with two nucleotides repeating eight times and having one nucleotide at 3' end as anchor. The list of ISSR and rRNA gene specific primers used in present study is shown in Tables 1 and 2.

PCR amplifications

The PCR amplification using ISSR primers was performed with following conditions: Denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min, for 35 cycles, followed by 10 min extension at 72 °C. Each 25 μl PCR reaction contained 2.5 mM MgCl₂, 200 μM dNTPs, 50 mM KCl, 10 mM Tris–HCl, 1% Triton X-100, 1 unit of Taq DNA polymerase, 5 pmol of primers, and 30 ng of genomic DNA. For multiplex PCR the annealing temperature was 52 °C when ISSR (AG)₅T repeat primer was used with pathogenic specific 16S rRNA gene primers and 55 °C when ISSR and primers of 23S rRNA genes were utilized.

| Table 1. Primer sequences of 23S rRNA and 16S rRNA gene. |
|-----|-----|-----|-----|
| 23S rRNA genus specific primers | 16S rRNA pathogenic serovar specific primers |
| Forward primer 5’ GACCGGAAGCTGAG 3’ | Forward primer 5’ CGCTGCCGGCGCTTTAAA 3’ |
| Reverse primer 3’ CATTAGGCTGATTGCGGCGC 5’ | Reverse primer 3’ AAGGTCACATGCCACCTT 5’ |

Results

Marker (AG)₅T-1000 is specific to the non-pathogenic species of *Leptospira*

DNA of standard non-pathogenic serovars, namely, patoc and ranarum belonging to *Leptospira* species *L. biflexa* and *L. meyeri* and of pathogenic serovars belonging to species, *L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. weilii* and *L. burgdorferi* were tested with ISSR-PCRs. Amplification of DNA could be observed only with primer (AG)₅T, wherein a region of 1000 bp was amplified in non-pathogenic species of *Leptospira*. Figure 1 shows the PCR-amplified product using primer (AG)₅T. The marker (AG)₅T-1000 was absent in the standard pathogenic serovars of the *Leptospira* species *L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. weilii* and *L. burgdorferi*. The ISSR-PCR using primer (AG)₅T was repeated a number of times to confirm the reproducibility of the amplified product. The primer (AG)₅T amplified the marker region in non-pathogenic species whereas other primers based on (AG) repeats with different anchors such as (AG)₅G and (AG)₅C did not produce amplification. This indicated that a single nucleotide anchor at 3’ end of the primer played an important role for specificity of PCR reaction in non-pathogenic *Leptospira* species.

Screening of Leptospira isolates from the patients and from the environmental samples

For evaluating the ISSR marker (AG)₅T-1000, 12 isolates of *Leptospira* recovered from hospitalized patients of PUO and/or from cases of febrile jaundice and 23 isolates obtained from different water sources were used. DNA was extracted from these 35 isolates and used for PCR analysis. PCR was performed using 23S rRNA genus specific primers which amplified 482 bp fragment from all the isolates, further confirming that these isolates belong to *Leptospira* species. All the isolates were then characterized with the conventional proce-