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Original Article

# *FlaB* PCR-based Identification of Pathogenic Leptospiral Isolates

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**BACKGROUND/PURPOSE:** The genus *Leptospira* comprises pathogenic and saprophytic strains. Conventional methods for the identification of pathogenic leptospiral isolates are cumbersome and laborious. In view of these limitations, the search for alternative methods have been focused on DNA based techniques. In this study, we have developed an effective method for the rapid identification of pathogenic and saprophytic leptospiral isolates based on DNA-based techniques.

**METHODS:** A polymerase chain reaction(PCR)-based approach was developed using specific primer sets (*flaB*, G1-G2, B64I-II, and A-B) to differentiate pathogenic and saprophytic leptospiral strains. Fifty-five leptospiral isolates were used for this study. The pathogenic status of the isolates was compared with the results obtained using conventional techniques, which included growth in the presence of 8-azaguanine and growth at 13°C.

**RESULTS:** In this analysis, 46 leptospiral isolates were confirmed as pathogenic and nine were confirmed as saprophytic. PCR with the A-B primer set yielded an amplified product of 331 bp in all of the pathogenic and saprophytic isolates. The other primer sets, G1-G2, B64I-II and *flaB*, yielded products of 258 bp, 568 bp, and 793 bp, respectively, exclusively for the pathogenic leptospiral strains. None of the saprophytic strains yielded products with these primer sets.

**CONCLUSION:** The *flaB*-specific primers consistently yielded an amplification product for all of the pathogenic leptospiral isolates, indicating the presence of the *flaB* gene only among pathogenic leptospires, and making this a useful tool for distinguishing between pathogenic and saprophytic leptospires. The efficiency of PCR-based identification corroborates the implementation of these techniques for the identification of pathogenic and saprophytic leptospiral strains.

**KEYWORDS:** 8-azaguanine, G1-G2, B64I-II, *flaB,* PCR, pathogenic leptospires

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## Introduction

Leptospirosis is considered the most widespread zoonotic disease in the world, occurring in a variety of urban and rural settings. Leptospirosis is caused by infection with pathogenic *Leptospira* species and can frequently lead to life-threatening disease in humans. It is characterized by hematogenous dissemination of the bacteria to multiple organs including the brain, aqueous humor, liver, lungs,

and kidneys.[1] Heterologous *Leptospira* species can cause disease and more than 300 serovars of *Leptospira interrogans* sensu lato and 45 serovars of *Leptospira biflexa* sensu lato have been described.[2] These pathogenic leptospires are responsible for human/animal infections. The pathogenic mechanisms of leptospires are not clearly defined but potential virulence factors include hemolysins, glycolipoproteins, heat shock proteins, and flagella.[3]

The saprophytic leptospires are indigenous to fresh surface water and to date, a clear parasitic or saprophytic role has not been established for these organisms. Mammals are not susceptible to experimental infection by these leptospires.[4] These pathogenic leptospires commonly inhabit the mammalian kidney and organisms are therefore excreted through urine. The presence of pathogenic leptospires in streams and water bodies is an index of leptospirosis in wildlife or domestic animals having access to these waters. Differentiation of pathogenic from saprophytic leptospires is important to classify the pathogenic status of the leptospires for epidemiological and taxonomical purposes.

Recently, species-specific primers which amplify the 16S and 23S rRNA regions and a portion of the *flaB* gene coding for the flagellar protein have been developed for the identification of pathogenic and saprophytic leptospires.[5–7] In an earlier study, the leptospiral strains belonging to similar serovars recovered from patients presenting with different clinical manifestations were compared using the randomly amplified polymorphic DNA fingerprinting technique, to understand the clonal relatedness and distribution of these strains.[8] The standard methods available for the differentiation of pathogenic from saprophytic leptospires are growth in the presence of 8-azaguanine and growth at 13°C.

In this study, polymerase chain reaction (PCR) amplification was performed using four primer sets (*flaB*, G1-G2, B64I-II and A-B) on the leptospiral isolates recovered from various sources to distinguish the pathogenic and saprophytic leptospires. The results were compared with those obtained using the standard methods of growth in the presence of 8-azaguanine and growth at 13°C.

## Methods

### *Leptospira strains*

A total of 55 leptospiral isolates, of various serovars, recovered from different sources, were included in this study. All of the strains were maintained in the leptospiral repository of the Regional Medical Research Centre, Port Blair, Andaman Islands, India with periodical subculture in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. Commercial EMJH (Difco, Detroit, Michigan, USA) medium was used, with the addition of 0.2% agarose (Sigma, St. Louis, MO, USA), 1% bovine serum albumin (Sigma), 2% rabbit serum, 0.1% sodium pyruvate (Merck, Germany) and 100 μg/mL 5-flurouracil (Merck) as a selective agent.

### *Isolation of leptospires from humans*

Individual consent was obtained from patients or their guardians and ethical clearance for sample collection was obtained from the ethical committee of the Regional Medical Research Centre. Blood samples (1–2 drops) from clinically suspected human cases of leptospirosis were immediately used to inoculate EMJH semisolid medium. Urine samples were processed according to standard procedures.[9] Urine samples (1–4 drops) were used to inoculate EMJH semisolid medium in McCartney bottles, with a hole cut in the aluminum cap and a rubber lining placed underneath.[10]

### *Isolation of leptospires from rat kidney*

Trapped field rats (*Rattus norvegicus*) were sacrificed by cervical dislocation, then washed in cetrimide solution. The body cavity was opened aseptically and a piece of kidney was extracted using rat toothed forceps and used to inoculate media in tubes, as described previously.[11]

### *Isolation of leptospires from water*

Water samples collected from endemic areas were centrifuged at 5000*g* and 1–3 mL of the supernatant was filtered through 0.22 μm nitrocellulose membrane filters (Millipore, Cork, Ireland) and aseptically added to EMJH medium.[4] All of the samples were inoculated into three sets of EMJH semisolid media tubes and the tubes were incubated at 30°C in the dark. The tubes were examined at weekly intervals by dark field microscopy for the presence of leptospires. Tubes showing evidence of growth were subcultured into fresh EMJH semisolid media vials and further monitored.

### *Growth of leptospires with 8-azaguanine*

A total of 0.5 mL of 8-azaguanine solution (2.25 mg/mL; Sigma) was added aseptically to 4.5 mL of EMJH media and mixed thoroughly. The tubes were then inoculated

with 0.5 mL of a well-grown culture of the test strain along with the controls in duplicate. The tubes were incubated at 30°C and examined twice a week up to 21 days.[12] For growth at 13°C, 0.5 mL of the strain under investigation was subcultured in 4.5 mL of EMJH medium in duplicate. Then the tubes were incubated at 13°C and examined twice a week up to 21 days.[13] A pathogenic strain (RGA) and a saprophytic strain (Patoc I) were included as controls.

### *Serovar level identification of the isolates*

A microscopic agglutination test using group sera was applied for the serogroup level identification and monoclonal antibodies (mAbs) of specific serovars were used for the serovar level identification. Both techniques were performed as previously described.[1] A panel of 37 "group sera" (rabbit antisera) representative of all pathogenic serogroups were used. To determine the serovar status of isolates, a panel of mouse mAbs (WHO/FAO Collaborating Centre for Reference and Research, KIT-Biomedical Research, Amsterdam, The Netherlands) belonging to serogroups: Grippotyphosa (F71C3, F71C9, F165C3, F165C8); Icterohaemorrhagiae (F52C1, F70C4, F70C14, F70C20, F70C24); Autumnalis (F69C15, F64C10, F69C11, F69C9); Australis (F81C1, F81C8, F90C5, F90C6); Sejroe (F13C193, F106C53, F16C28, F21C2); Hebdomadis (F50C3, F106C5); Javanica (F20C4, F98C8, F98C12, F98C19, F98C20); Pyrogenes (F134C6); Pomona (F46C9, F46C6); and Canicola (F152C11, F152C14, F152C17, F152C18) were used.

### *Preparation of genomic DNA*

Genomic DNA was extracted and purified according to the method described previously.[14] Exponentially growing *Leptospira* cultures were centrifuged at 12,000*g* for 30 minutes at 4°C. The pellet was resuspended in Tris-EDTA buffer (10 mM Tris, 10 mM $MgCl_2$, 2 mM EDTA, 400 mM NaCl, and 10 mM KCl) with lysozyme (5 mg/mL) and proteinase K (10 mg/mL) and then lysed in CTAB/NaCl solution (CTAB, 270 mm; NaCl, 700 mm). The mixture was then subjected to chloroform-isoamyl alcohol extraction and ethanol precipitation. After washed twice with 70% ethanol, the pellet was dried and then dissolved in Milli-Q water and used for PCR analysis.

### *PCR amplification*

PCR was carried out in a 50 µL reaction mixture, contained 50 ng of purified DNA, 0.1 µM of each primer, 250 µM of dNTP (Genei, Bangalore, India), 3 mM of $MgCl_2$, 0.5 U of Taq DNA Polymerase (NEB, MA, USA), in 10 mM Tris-HCl (pH 9) and 50 mM KCl. Four sets of primers were used in the PCR, according to the standard procedures described previously.[5–7] Primers sequenced were listed in Table 1. Initiation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at specific temperature for each primer pair for 59 seconds (Table 1), extension at 72°C for 1 minute, and final extension at 72°C for 7 minute. PCR was performed in a DNA Engine PTC 200 thermal cycler (MJ Research Inc., USA). The PCR products were electrophoresed in a 1.5% agarose gel along with a 100 bp DNA ladder (Bangalore, Genei, India) in TAE buffer containing 0.5 µg/mL ethidium bromide. The PCR products were viewed under UV illumination and documented using a gel documentation system (Bio-Rad, USA).

**Table 1.** Primer sequences used in this study[5–7]

| Gene | Primer | Sequences | Annealing (°C) |
|---|---|---|---|
| G1-G2 | forward | 5′-CTG AAT CGC TGT ATA AAA GT-3′ | 60 |
| | reverse | 5′-GGA AAA CAA ATG GTC GGA AG-3′ | |
| B64I-II | forward | 5′-CTG AAT TCT CAT CTC AAC TC-3′ | 60 |
| | reverse | 5′-GCA GAA ATC AGA TGG ACG AT-3′ | |
| *flaB* | forward | 5′-TCT CAC CGT TCT CTA AAG TTC AAC-3′ | 59 |
| | reverse | 5′-CTG AAT TCG GTT TCA TAT TTG CC-3′ | |
| A-B | forward | 5′-GGC GGC GCG TCT TAA ACA TG-3′ | 62 |
| | reverse | 5′-TTC CCC CCA TTG AGC AAG ATT-3′ | |

## Results

In total, 55 leptospiral isolates from various sources, including human blood ($n=40$), human urine ($n=2$), rat kidney ($n=5$), cow urine ($n=1$), dog blood ($n=2$), and water bodies ($n=5$) were used for this study. The isolates from human specimens were collected from patients with suspected leptospirosis. Serovar level identification of the isolates was carried out using 37 group sera in a cross agglutination test and was further confirmed by serovar specific mAbs using a microscopic agglutination test. The serovar status of the pathogenic and the unclassified non-pathogenic isolates can be seen in Tables 2 and 3. The mAb patterns observed for the pathogenic isolates are presented in Figure 1. Of the 55 isolates obtained, 22 belonged to serogroup Grippotyphosa, six to serogroup Pomona, five to serogroup Icterohaemorrhagiae, five to serogroup Canicola, three to serogroup Australis, and one belonged to each of the following serogroups: Pyrogenes, Sejroe, Autumnalis, Hebdomadis, and Javanica. The remaining nine were unclassified. The pathogenic status of the isolates were initially determined by conventional techniques, such as growth in the presence of 8-azaguanine and growth at 13°C. Inoculated tubes were incubated up to 21 days and the tubes were observed at days 0, 7, 14, and 21, and the number of leptospires was recorded under dark field microscopy with a magnification of (10× and 20×).[15] The maximum number of leptospires observed for pathogenic isolates was in the range of 10–150, during the 21-day incubation period, and for saprophytic isolates it was in the range of 400–1,020 organisms/field. When comparing the standard procedure results with the group sera analysis, 46 isolates were classified as pathogenic and nine as saprophytic. The saprophytic isolates did not react with any of the 37 group sera of the pathogenic serovars representing the 23 serogroups in the cross agglutination test and were categorized as unclassified isolates. The saprophytic isolates also did not show any agglutination with the hyper immune sera raised against the saprophytic strain Patoc I.

Apart from the conventional identification methods, PCR-based methods were also employed using specific primers for the *flaB* gene, the G1-G2 region, the B64I-II region and the A-B region, and the product sizes are shown in Figure 2. The leptospiral *flaB* gene was detected in all of the 46 pathogenic isolates, the G1-G2 region amplified in 44 isolates and the B64I-II region amplified in two isolates. A 793 bp fragment was amplified using the *flaB*-specific primers in the pathogenic leptospiral isolates. Amplification of the A-B region yielded a product of 331 bp in all of the isolates, which corresponded to nucleotides 38–57 and 348–368 in the *L. interrogans* 16S rRNA gene. These primer sets therefore amplified a specific portion of the DNA invariably from all of the pathogenic and saprophytic leptospires.[6] The G1-G2 primers amplified a product of 285 bp from all of the pathogenic isolates of non-*Leptospira kirschneri* groups whereas the B64I-II primers yielded a product of 563 bp from *L. kirschneri.* Interestingly, the strains shown to be pathogenic leptospires by conventional techniques were confirmed by the PCR-based approaches. As with the PCRs for the G1-G2 and B64I-II regions, the *flaB* PCR yielded a consistent amplification product with all pathogenic strains, indicating conservation of this gene amongst pathogenic leptospires.

## Discussion

Conventional methods for the identification of pathogenic leptospiral isolates are time consuming and laborious and there is, therefore, a need for a rapid and simple molecular based identification method as an alternative. In earlier studies, a single set of G1-G2 primers along with A-B primers were used for the identification of pathogenic leptospires.[16] However, this approach does not accurately detect *L. kirschneri* strains and may wrongly classify pathogenic strains of *L. kirschneri* as saprophytic strains. This may be overcome by using another set of primers, B64I-II, which specifically amplify *L. kirschneri*. In southern India, *L. kirschneri* is one of the prevailing species and therefore a multiplex PCR, including both the G1-G2 and B64I-II primer sets, is essential for accurate identification of leptospires.[18] In one study, multiplex PCR was performed on urine samples collected from patients, but they focused on diagnosis rather than identification of the species.[17] In another study, a nested PCR-restriction fragment length polymorphism assay was developed for the identification of the predominant pathogenic species in clinical samples for the early diagnosis of leptospirosis.[19] Recently, many techniques have been applied to the identification of *Leptospira* species, such as randomly amplified polymorphic DNA fingerprinting and a three-step procedure with

**Table 2.** Serovar and the pathogenic status of the leptospiral isolates

| Sample | Isolate | Serovar | Source | Pathogenic status |
|---|---|---|---|---|
| 1 | MG347 | Australis | Human blood | Pathogenic |
| 2 | AHF651 | Australis | Human blood | Pathogenic |
| 3 | MG392 | Australis | Human blood | Pathogenic |
| 4 | N2 | Autumnalis | Human urine | Pathogenic |
| 5 | PAI | Canicola | Human urine | Pathogenic |
| 6 | H12 | Canicola | Human blood | Pathogenic |
| 7 | IAH | Canicola | Human blood | Pathogenic |
| 8 | D14 | Canicola | Dog blood | Pathogenic |
| 9 | D7 | Canicola | Dog blood | Pathogenic |
| 10 | H22 | Grippotyphosa | Human blood | Pathogenic |
| 11 | DS15 | Grippotyphosa | Human blood | Pathogenic |
| 12 | DCHCF30 | Grippotyphosa | Human blood | Pathogenic |
| 13 | ALC10 | Grippotyphosa | Human blood | Pathogenic |
| 14 | R41 | Grippotyphosa | Rat kidney | Pathogenic |
| 15 | R42 | Grippotyphosa | Rat kidney | Pathogenic |
| 16 | D22 | Grippotyphosa | Human blood | Pathogenic |
| 17 | MG472 | Grippotyphosa | Human blood | Pathogenic |
| 18 | DS18 | Grippotyphosa | Human blood | Pathogenic |
| 19 | BL10 | Grippotyphosa | Human blood | Pathogenic |
| 20 | MG670 | Grippotyphosa | Human blood | Pathogenic |
| 21 | MG100 | Grippotyphosa | Human blood | Pathogenic |
| 22 | MG23 | Grippotyphosa | Human blood | Pathogenic |
| 23 | MG11 | Grippotyphosa | Human blood | Pathogenic |
| 24 | TB19 | Grippotyphosa | Human blood | Pathogenic |
| 25 | Thankachan | Grippotyphosa | Human blood | Pathogenic |
| 26 | H2 | Grippotyphosa | Human blood | Pathogenic |
| 27 | Mg373 | Grippotyphosa | Human blood | Pathogenic |
| 28 | Mg663 | Grippotyphosa | Human blood | Pathogenic |
| 29 | MG569 | Grippotyphosa | Human blood | Pathogenic |
| 30 | GC1 | Grippotyphosa | Human blood | Pathogenic |
| 31 | TB6 | Grippotyphosa | Human blood | Pathogenic |
| 32 | ALC1 | Hebdomadis | Human blood | Pathogenic |
| 33 | AF61 | Icterohaemorrhagiae | Human blood | Pathogenic |
| 34 | GC3 | Icterohaemorrhagiae | Human blood | Pathogenic |
| 35 | AHFY | Icterohaemorrhagiae | Human blood | Pathogenic |
| 36 | APSK1 | Icterohaemorrhagiae | Rat kidney | Pathogenic |
| 37 | APSK2 | Icterohaemorrhagiae | Rat kidney | Pathogenic |
| 38 | R1 | Javanica | Rat kidney | Pathogenic |
| 39 | H578 | Pomona | Human blood | Pathogenic |
| 40 | MG39 | Pomona | Human blood | Pathogenic |
| 41 | 289MC | Pomona | Human blood | Pathogenic |
| 42 | H3 | Pomona | Human blood | Pathogenic |
| 43 | L36 | Pomona | Human blood | Pathogenic |
| 44 | H48 | Pomona | Human blood | Pathogenic |
| 45 | DrGhoshA | Pyrogenes | Human blood | Pathogenic |
| 46 | AHF421 | Sejroe | Human blood | Pathogenic |

**Table 3.** Pathogenic status of the unclassified leptospiral isolates

| Sample | Isolate | Serovar | Source | Pathogenic Status |
|---|---|---|---|---|
| 1 | G3 | Unclassified | Human blood | Non-pathogenic |
| 2 | G6 | Unclassified | Human blood | Non-pathogenic |
| 3 | W41 | Unclassified | Pond water | Non-pathogenic |
| 4 | DrGhoshB | Unclassified | Human blood | Non-pathogenic |
| 5 | WTS1 | Unclassified | Pond water | Non-pathogenic |
| 6 | WTS2 | Unclassified | Pond water | Non-pathogenic |
| 7 | WT62 | Unclassified | Pond water | Non-pathogenic |
| 8 | WT11 | Unclassified | Pond water | Non-pathogenic |
| 9 | C5 | Unclassified | Cow urine | Non-pathogenic |

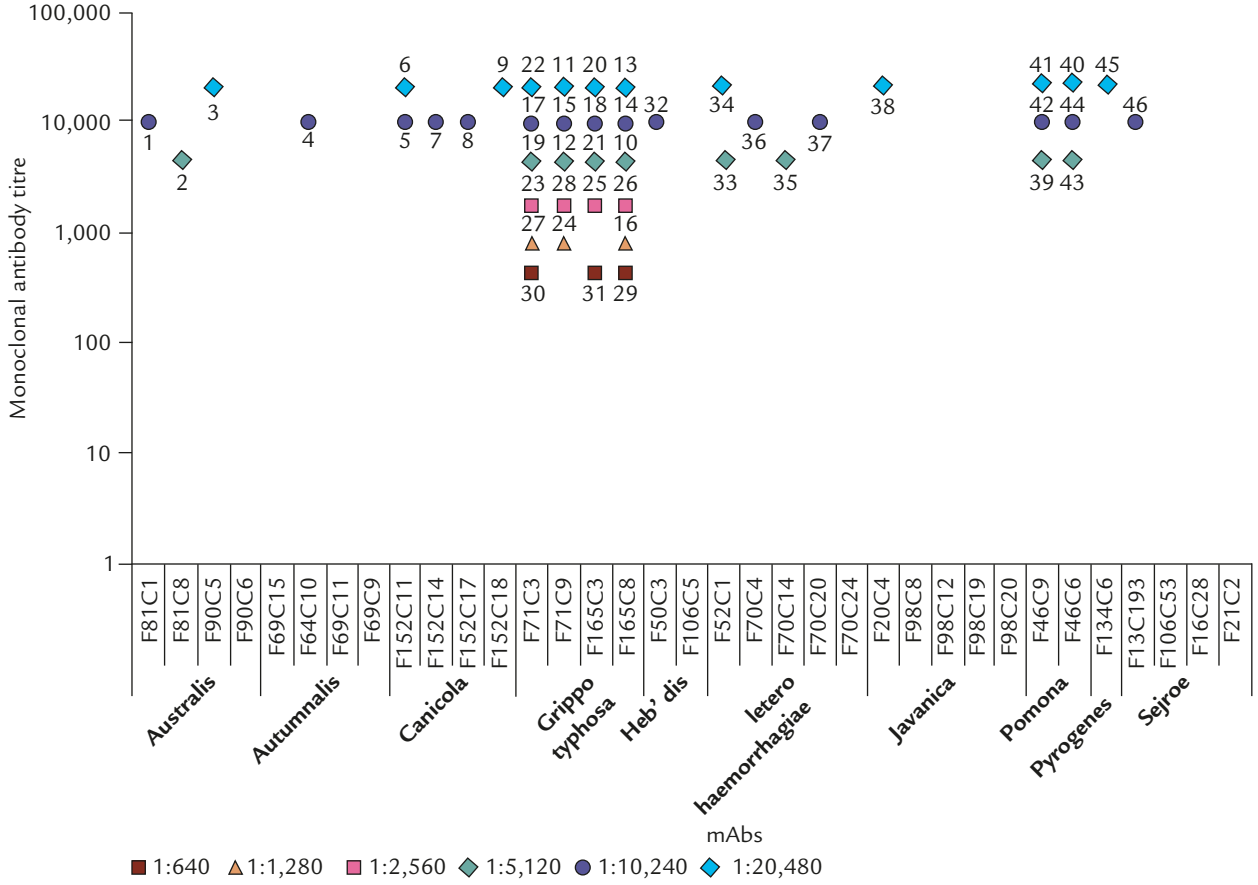


**Figure 1.** The monoclonal antibody pattern of the pathogenic leptospiral isolates.

amplified fragment length polymorphism,[20] but all of these approaches require a high degree of expertise for analysis of the results. Furthermore, these molecular-based techniques only determine the species and not the serovar status of the isolates. Therefore, we established a PCR assay that included the *flaB* primers as well as the A-B primers, to simplify the experimental approach for identifying pathogenic *Leptospira* species. The *flaB* primers only amplify a specific fragment from pathogenic leptospires, allowing rapid identification of pathogenic isolates. The G1-G2 and B64I-II primer sets can be used in both single and multiplex PCR (data not shown). The A-B primer set amplifies a DNA fragment from both pathogenic and saprophytic leptospires. Together with the *flaB* PCR result, the

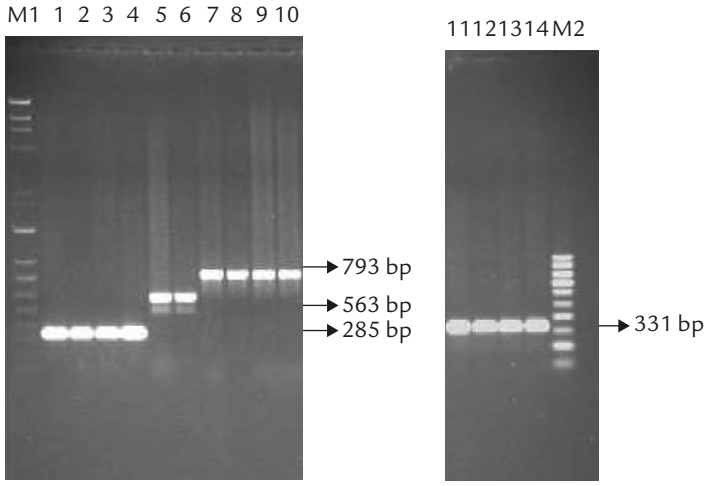


**Figure 2.** PCR amplified fragments of *flaB*, G1-G2, B64I-II, and A-B. M1, λ DNA/*Hin*dIII/pUC18/*Sau*3AI-pUC18/*Taq*I digest; lanes 1–4, G1-G2 product of H578, MG39, AF61, and N2; lanes 5–6, B64I-II product of DCHCF30, and ALC10; lanes 7–10, *flaB* product of H578, MG39, AF61, and N2; lanes 11–14, A-B product of H578, MG39, AF61, and N2; and M2, 100 bp DNA ladder.

pathogenic leptospires can be distinguished from the saprophytic leptospires. To conclude, the *flaB* PCR-based approach is an effective method for the rapid preliminary identification of the pathogenic nature of leptospiral isolates.

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