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ORIGINAL ARTICLE

Application of pulsed-field gel electrophoresis for molecular identification of pathogenic *Leptospira* species in Iran: a rapid and reliable method

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ABSTRACT

Background and Objectives: Leptospirosis is a zoonotic disease caused by pathogenic *Leptospira* serovars. The genus *Leptospira* cannot differentiated by conventional techniques. However, identity determination of pathogenic serovar is precious of public health problems and epidemiological studies. Pulsed-field gel electrophoresis facilitates rapid identification of *Leptospires* to the serovar levels.

Materials and Methods: In this study, we employed PFGE to evaluate 28 *Leptospira* isolates, with animal, human and environmental origin, obtained from Razi Vaccine and Serum Research Institute of Karaj, Iran. PFGE patterns of 28 *Leptospira* serovars were generated using the *Not* I restriction enzyme in comparison with the lambda ladder.

Results: Out of 28 serovars evaluated, we identified 22 different pulsed types, designated P1- P22. Out of 22 pulse groups, 3 were found to be a common type, but others were a single Type. Groups consisting of the common type were P3, P9, P14, and P16. The results showed that the discriminatory index of PFGE by *Not* I enzyme was 0.99, demonstrating heterogeneous differentiation among serovar members.

Conclusion: The PFGE methodology used in this study showed excellent interlaboratory report usability, rapid, reliable, enabling standardization and data sharing between laboratories.

Keywords: Leptospirosis; Pulsed-field gel electrophoresis; *Leptospira* serovars; Molecular typing method

INTRODUCTION

Leptospirosis is a zoonotic infection and public health problem worldwide (1, 2). Humans and animals become infected through directly and indirectly contact with the contaminated urine of infected animals (3, 4). It is important to note that human cases of *Leptospira* infection can be severe, leading to multi-organ failure in individuals who were previously healthy (5). In the past, *Leptospira* was classified into two species, *L. interrogans* and *L. biflexa*, based on serological methods. *L. interro-*

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gans contained pathogenic serovars, while *L. biflexa* contained saprophytic serovars. Recently, the *Leptospira* genus is now classified into 69 genomic species (including pathogenic and saprophytic species) based on whole genome sequencing and DNA-DNA hybridization method (6, 7). There are more than 260 pathogenic and 60 saprophytic serovars (8, 9), and therefore identification of *Leptospira* serovars is essential for epidemiological studies of leptospiral infections. This can lead to the detection of infected hosts, and it is valuable to identify new species or serovars (10, 11). However, identification of *Leptospira* is complicated, because different serovars are divided among several species, presumably due to horizontal gene transfer (11). Due to the difficulties, serovar characterization is only performed in reference laboratories (12). Detection of *Leptospira* serovars typically involves the use of large panels of reference antisera and live antigens, which can be time-consuming and require specialized laboratory expertise. An alternative and more efficient method for identifying and standardizing *Leptospira* serovars is Pulsed-field gel electrophoresis (PFGE). This method provides a more accurate and efficient way to identify and standardize *Leptospira* serovars compared to traditional methods that rely on reference antisera and live antigens (12, 13). The method also has a potential ability to differentiate among strains of serovars belonging to diverse species. Until now, there is no epidemiological analysis of *Leptospira* isolates by genotypic methods in Iran. To this end, the present study set out to determine the potential utility of the PFGE method for the differentiation of *Leptospira* strains.

MATERIALS AND METHODS

Bacterial isolates. A total of 28 *Leptospira* isolates, with animal, human and environmental origin, obtained from Razi Type Culture Collection (RTCC), *Leptospira* reference laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran (Table 1). The Bacterial isolates were inoculated into the liquid EMJH medium (Difco, USA) at 28-30°C for 7-10 days (13), and then centrifuged at 17000 ×g for 15 minutes and then resuspended in 1 ml of the 1 × TE buffer (100 mM Tris, pH 7.5, and 100 mM EDTA, pH 8) to OD_{610} of 1.200 calculated using a spectrophotometer (Ultrospec 2000, pharmasia, England).

Table 1. Bacteria used in this study

No.	RTCC No.	Serogroup	Serovar
1	2802	Interogans	Autumnalis
2	2805	Interogans	Canicola
3	2808	Interogans	Grippotyphosa
4	2810	Interogans	Sejro hardjo
5	2812	Interogans	Icterhaemorhagiae
6	2815	Interogans	Pomona
7	2817	Interogans	Sejro serjoe
8	2818	Interogans	Semanerga
9	2821	Interogans	Sejro hardjo
10	2822	Interogans	Pomona
11	2823	Interogans	Icterhaemorhagiae
12	2824	Interogans	Canicola
13	2825	Interogans	GrippotypHosa
14	2828	Interogans	Semanerga
15	2829	Interogans	Pomona
16	2830	Interogans	Autumnalis
17	2831	Interogans	Malaysia
18	2832	Interogans	Celledoni
19	2833	Interogans	Lyme
20	2834	Interogans	Diasiman
21	2835	Interogans	Pyrogenes
22	2836	Interogans	Canicola
23	2837	Interogans	Icterhaemorrhagiae
24	2838	Interogans	Ballum
25	2839	Interogans	Javanica
26	2840	Interogans	Australis
27	2841	Interogans	Lai type Lanylokowii
28	2842	Interogans	Bataviae

Preparation of agarose plugs. To prepare samples for PFGE analysis, 10 µl of proteinase K (20 mg/mL) was added to 200 µL of bacterial suspension, followed by an equal volume of molten plug agarose solution (Sigma, USA). The mixture was then dispensed into wells of a disposable plug mold and allowed to solidify for about 10-15 minutes. The Agarose plugs were then immersed in 2 mL of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, pH=8.0, 1% N-lauroylsarcosile, 20 mg/mL proteinase K) and incubated in a water bath at 55°C for 90 minutes. After incubation, the plugs were transferred to a tube containing 4 mL of sterile distilled water and incubated for an additional 20 minutes in a water bath at 50°C. The plugs were then washed three times in wash buffer (10 mM Tris, 1 mM EDTA, pH=8.0) at 50°C for 20 minutes each (14).

Restriction enzyme digestion and PFGE. Agarose plugs were cut into several slices, and the DNA-embedded slice was digested with 30U of *Not* I (Fermentas, England) in a water bath at 37°C for 3 hours. DNA size marker was New England Biolab lambda ladder. The Plug slices containing the digested DNA were subjected to electrophoresis in CHEF-DRIII (Bio-Rad Laboratories) with recirculating 0.5× Tris-borate EDTA (TBE) buffer for 22 hours at 14°C. The electrophoresis experiment was conducted under specific conditions: switch times of 2.16 and 35.07 seconds, an angle of 120°, gradient of 6 v/cm, at 14°C, and duration of 22 hours. Following electrophoresis, the gels were stained with ethidium bromide (1 µg/mL) from Sigma (USA) and then destained in water for 20 minutes. Fragment patterns were analyzed by visualizing the gels on a UV Tran illuminator and capturing photographs using an imaging system (Gel Doc 2000 System, Bio-Rad Laboratories). Gel analysis and dendrogram were generated using gel compare II software. Patterns with identical fragments and molecular weights were considered indistinguishable. Patterns with differences greater than 75%, between 45-75%, and less than 45% were classified as closely related genetically, possibly related, and different, respectively.

RESULTS

PFGE profiles were generated from 28 different *Leptospira* isolates. PFGE patterns (pulse types) of *Leptospira* serovars were generated from *Not* I restriction enzyme (P1 to P22). Digestion of genomic DNA with *Not* I restriction enzyme resulted in a 9-24 band and restriction fragments were found to be ranging from 40 to 1,100 kb (Fig. 1). Out of the 28 serovars, 22 pulse types were observed.

The discrimination index was high for PFGE (DI=0.99). The similarity range was between 40-100% (Fig. 1). Out of 28 pulse types, 4 types, including P8, P9, P14, and P16, exhibited more than one isolate. Three pulse types, including P8, P9, and P16, showed the same serovars while one pulse type, P14, had isolates with different serovars. One pulse type with three same isolates of *L.* Canicola (P9) and one pulse type with three different isolates (P14) were shown to be the largest pulse types in our isolates. In contrast, other pulse types had only one isolate.

To investigate reproducibility of PFGE, two samples in one gel with two different positions were tested, leading to similar ranges and bands. Results from this study showed that this technique is reproducible, and has the ability to distinguish between isolates as well as serotypes.

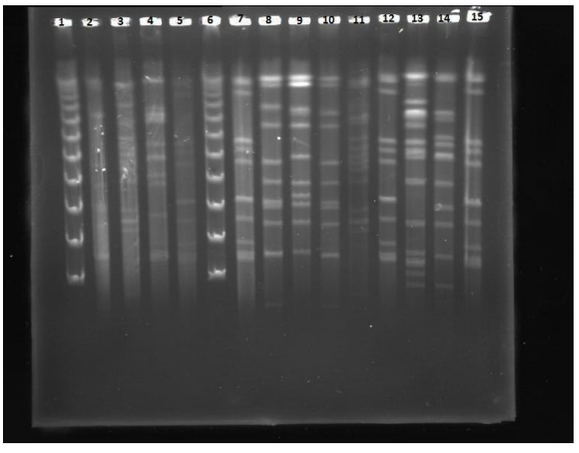

Fig. 1. Representative PFGE-*Not* I profiles of *leptospira* isolates.

Lane 1and 6. Lambda ladder PFGE marker (N0340S, New England Biolab, USA; band size: 48.5-1018.5 kb), lane 2. *L.* Conicola (2805), lane 3. *L.* Grippotyphosa (2808), lane 4. *L.* Sejro hardjo (2810), lane 5. *L.* Icterhaemorhagiae (2812), lane 7 and 15. *L.* Semanerga (2819), lane 8. *L.* Sejro hardjo2821), lane 9. *L.* Pomona (2822), lane 10. *L.* Canicola (2824), lane 11. *L.* Grippotyphosa (2825), lane 12. *L.* Pomona (2829), lane 13. *L.* Diasiman (2834), lane14. *L.* Autumnalis (2840).

DISCUSSION

Leptospirosis is a human and veterinary public health problem. Infection with pathogenic *Leptospira* occurs by exposure to contaminated watered environments. In addition, the disease is traditionally associated with occupational contexts (1, 2). The most common pathogenic serovars of *Leptospira* that cause infection in humans are *L.* Icterohaemorrhagiae, *L.* Grippotyphosa, and *L.* Canicola. In cattle, the *L.* Serovars serjoe hardjo and *L.* Grippotyphosa are prevalent, while in other animals *L.* Canicola, *L.* Icterohaemorrhagiae, and *L.* Grippotyphosa are commonly found (15, 16). Classifying *Leptospira* isolates into serovars is crucial for understanding the epidemiology of leptospirosis, identifying reservoir hosts, and implementing targeted prevention measures to control outbreaks and identification of new serovars

(17, 18). Traditional phenotypic typing methods have become less effective due to the increased diversity of *Leptospira* isolates. Accordingly, molecular typing methods are now being used to differentiate isolates in the *Leptospira* serovars (10, 18). Some methods such as the microscopic agglutination test (MAT), are laborious, time consuming and require the maintenance of different serovars (19). Some molecular methods like multilocus sequence typing (MLST) and variable-number tandem repeats (VNTR) analysis require specific primers for each species and cannot easily distinguish between serovars at a subserovar level. Therefore, such methods cannot be applied easily to all isolates (12). PFGE is commonly used for surveillance of a wide range of bacteria due to its high discriminatory power (20). By this technique as a standard molecular typing method, rapid identification of *Leptospires* is now possible. The potential to identification of new serovars or species using PFGE is also significant (10, 21). Moreover, PFGE serves as a complementary method that produces easily interpretable fingerprints compared to serological methods (22). This method is particularly valuable due to its reproducibility and ability to distinguish between *Leptospira* serovars (23). There are no or few data about molecular epidemiological analyses of *Leptospira* spp. using the PFGE method in Iran. Therefore, our aim was to assess molecular typing of *Leptospira* isolates using the PFGE typing method.

In the present study, 22 different patterns observed among 28 isolates. *Not* I restriction enzyme produced fragment patterns consisting of 9-24 bands ranging from 40 to 1100 kb. The maximum fragments were for *L*. Lyme (2833) with 24 bands. The minimum fragments were found in *L*. Icterhaemorhagiae (2823) with 9 bands.

The results revealed the most of PFGE patterns were unique type (Fig. 2). In our study, one cluster, P9, was shown to be one of the two largest pulse types, which contained three same isolates of *L*. Canicola. They shared the same serotype and source. On the other hand, one cluster, P14, contained three different isolates (Icterhaemorhagiae, Semanerga, and Autumnalis), showing a similar pattern. This can be because of indistinguishable profiles resulting from either cross-contamination or the deficiency of

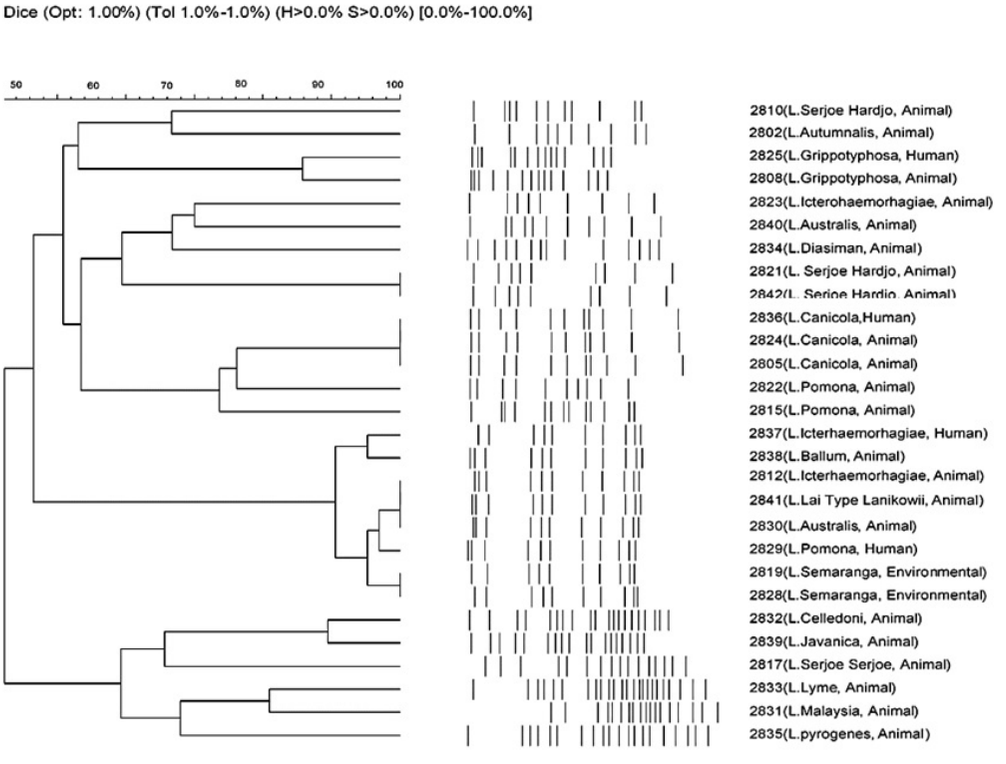

Fig. 2. The dendrogram illustrating the relationship between 28 *Leptospira* isolates from humans and animals in Iran, representing 28 serotypes, was generated using gel compare II software. PFGE profiles were obtained for clusters containing multiple isolates. Similarities between strains were assessed using the Dice coefficient and the UPGMA clustering method.

discriminatory power of this technique. There was a high genomic diversity between *L*. Icterhaemorhagiae (2812, 2823, and 2837) and *L*. Pomona (2815, 2822, and 2829) as the 3 isolates were subtyped into 3 pulse types. Each showed different profiles in the pattern and unrelated clonal isolates. Although belonging to the same serotype and source, they were not in the same pulse group. Previous researches have demonstrated that repeated instances of same serovar incessantly yield reproducible profiles, and PFGE types of various strains within the same serovar are nearly linked (14, 17).

In a 2008 study, Galloway et al. employed a modified PFGE technique to identify *Leptospira* serovars. They found that PFGE can quickly identify the epidemiologically unrelated isolates and able to substitute laborious serologic identification tests. These findings were consistent with our results (12).

In Brazil, Romero et al. in 2009 applied pulsed-field gel electrophoresis to discriminate between leptospiral isolates. Their results indicated that PFGE could be a valuable tool for quickly identifying Leptospires, which is necessary for developing disease prevention strategies. Furthermore, they reported that PFGE is a completed method with great ability to produce fingerprints that are easier than serological methods (22). These two results agreed with those obtained in our study.

Our study confirmed that PFGE is a dependable and reproducible technique for typing *Leptospira* strains, with a discrimination index of 0.99. This indicates that PFGE has high discriminatory power in distinguishing between *Leptospira* serovars. Overall, our findings support the use of PFGE as an efficient, rapid, and reliable method for epidemiological studies on *Leptospira* isolates.

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