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Original article

Fingerprints by pulsed-field gel electrophoresis of leptospires isolated from field rats and comparison with reference *Leptospira* serovars

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Background: More than 260 leptospiral serovars by serology have been recorded. New genotypic methods have classified *Leptospira* into 20 species. Serovar identification is essential for epidemiological study of this disease.

***Objective*:** We identified fingerprints from reference serovar strains of *Leptospira* spp. and representative serovars from field rat isolates by pulsed-field gel electrophoresis (PFGE).

***Methods*:** Extract genomic DNA from 28 reference serovars of *Leptospira* spp. and 13 leptospiral isolates from field rats, selected as representative serovars Pyrogenes, Bataviae, Autumnalis, and Australis, using PFGE following *Not*I restriction digest.

***Results*:** PFGE with *Not*I restriction enzyme successfully differentiated 28 reference serovars into 27 fingerprint patterns, with the exception of serovars Copenhageni and Icterohaemorrhagiae. The discriminatory power of these reference strains was 0.99. Isolates that yielded patterns identical to their corresponding serovars were serovars Pyrogenes strain Salinem and Bataviae. Patterns for isolates of serovars Autumnalis and Australis were different from reference serovar Autumnalis strain Akiyami A and serovar Australis strain Ballico used in this study.

Conclusion: PFGE can be useful for identifying serovars of leptospiral isolates from reservoirs and for identifying new serovars of *Leptospira* for epidemiological study.

Keywords: *Leptospira*, *leptospiral* serovar, molecular typing, PFGE, pulsed-field gel electrophoresis

Leptospirosis is caused by spirochetes belonging to pathogenic members of the genus *Leptospira*. Infection in humans occurs through direct or indirect contact with urine of infected animals [1, 2]. Rats and other rodents are the most common reservoirs, which may transfer infection to domestic animals, and humans [2]. Thus, knowledge of the prevalent serovars with genetic characterization and their maintenance hosts is essential to understanding the epidemiology of this disease.

The taxonomy of *Leptospira* is still a work in progress. There are 2 systems to classify *Leptospira*. One system is based on a serotypic method, which classifies leptospires into serovars by agglutination after cross-absorption with homologous antigens [3]. More than 260 antigenically related serovars are grouped into 24 serogroups [4]. The other system is based on a genotypic method. Recently, the genus *Leptospira* was classified into 20 species based on DNA hybridization studies [5].

Traditionally, *Leptospira* was classified and identified into serovars by cross agglutinin absorption test (CAAT). This technique requires maintenance and handling of live leptospires, which is laborious and presents biosecurity risks [3]. DNA-based methods can also be used for identification and characterization of leptospires. These include DNA:DNA hybridization [6, 7], restriction endonuclease analysis (REA) [8], and arbitrarily primed PCR (AP-PCR) [9].

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Polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) method differentiates few serovars [10]. The random amplified polymorphic DNA method with requirement for purified DNA samples has shown some promise for differentiating individual serovars [11]. Ribotyping, using *Eco*RI for digestion and 16S and 23S rRNA from *Escherichia coli* as the probe gave unique profiles for many serovars [12]. Moreover, Southern blot ribotyping based on *Eco*RV and *Hin*dIII digestion with two 16S and 23S rDNA probes fribotyping was a quick and powerful tool for differentiating *Leptospira* serovars [13]. Pulsed-field gel electrophoresis (PFGE) also provides a reliable and reproducible identification of *Leptospira* serovars [14] and PFGE is considered the gold standard for determining the relatedness between strains of pathogens.

In this study, PFGE was used to characterize reference serovar strains and to compare results between leptospires isolated from field rats as representative serovars and reference leptospiral serovar strains.

Materials and methods

Leptospira reference strains

Among 28 reference serovars, 27 pathogenic *Leptospira* species included 18 *L. interrogans*, 4 *L. borgpetersenii*, 2 *L. weilii*, and 1 each of *L. kirscheneri*, *L. noguchii*, and *L. meyeri*. One saprophytic species was identified as *L. biflexa*. These reference serovar strains were provided by the Thai National Institute of Health (NIH) and the National Institute of Animal Health of Thailand (**Table 1**).

Table 1. Serogroup, serovar, strain, and species used in this study

	No.	Serogroup	Serovar	Strain	Species
Pathogenic Leptospira (n = 27)	1	Australis	Australis	Ballico	L. interrogans
	2		Bangkok	Bangkok D 92	
	3		Bratislava	Jez Bratislava	
	4		Autumnalis	Akiyami A	
	5	Autumnalis	New	Heusden P2062	L. interrogans
	6		Rachamati	Rachmat	
	7	Ballum	Ballum	MUS 127	L.borgpetersenii
	8	Bataviae	Bataviae	Van Tienam	L. interrogans
	9	Canicola	Canicola	Hound Utrech IV	L. interrogans
	10	Cellidoni	Cellidoni	Celledoni	L. weilii
	11	Djasiman	Djasiman	Djasiman	L. interrogans
	12	Grippotyphosa	Grippotyphosa	Moskva V	L. kirscheneri
	13	Hebdomadis	Hebdomadis	Hebdomadis	L. interrogans
	14	Icterohaemorrhagiae	Copenhegeni	M 20	L. interrogans
	15		Icterohaemorrhagiae	RGA	
	16	Javanica	Javanica	Veldrat Bataviae 46	L. borgpetersenii
	17	Louisiana	Saigon	L 79	L. interrogans
	18	Panama	Panama	CZ 214 K	L. noguchii
	19	Pomona	Pomona	Pomona	L. interrogans
	20	Pyrogenes	Pyrogenes	Salinem	L. interrogans
	21		Zanoni	Zanoni	
	22	Ranarum	Ranarum	ICF	L. meyeri
	23	Sarmin	Sarmin	Sarmin	L. weilii
	24	Sejroe	Hardjo	Hardjoprajitno	L. interrogans
	25		Sejroe	M84	L. borgpetersenii
	26		Wolffi	3750	L. interrogans
	27	Tarassovi	Tarassovi	Perepelicin	L. borgpetersenii
Saprophytic Leptospira	28	Semaranga	Patoc	Patoc I	L. biflexa

Representative leptospiral isolates from field rats

Thirteen *Leptospira* strains isolated from field rats in the northeastern region of Thailand between 1999 and 2000 were selected as representative serovars Pyrogenes, Bataviae, Autumnalis, and Australis. They were provided by the Armed Forces Research Institute of Medical Sciences (AFRIMS). Serovars of these isolates were identified and characterized by microscopic agglutination test (MAT), monoclonal antibody coated latex test, and cross absorption agglutination test (CAAT).

Culture conditions and preparation of chromosomal DNA

Leptospires were grown to a stationary phase in 7 to 14 days at 29°C in liquid medium Ellinghausen-McCullough-Johnson-Harris (EMJH) [15] with shaking. Genomic DNA was extracted by DNA detection kit (QIAamp DNA mini kit, Qiagen, Germany) according to the manufacturer's recommendations.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed as described previously [14] with some modification described by the Centers for Disease Control and Prevention, Atlanta, Georgia [16]. Briefly, leptospiral cultures containing 10^9 cells were pelleted by centrifugation and then resuspended in TE buffer. The mixture of an equal volume of the cell suspension with 1.6% certified low melt agarose (Bio-Rad Laboratories, U.S.A.), was immediately dispensed into a plug mold. After lysis with proteinase K, the agarose plugs containing intact *Leptospira* genomic DNA were washed and then stored in fresh Plug Wash Buffer at 4°C until used. Prior to digestion, the agarose plugs were cut into 2 to 3 mm thick pieces. The plug slices were placed in a fresh mixture containing 30 U of *Not*I restriction enzyme in 1X restriction buffer. The digestion was carried out at 37C for 8 hours. The plug slices were placed on the comb and then, 1% pulsed field certified agarose (Bio-Rad Laboratories, USA) was poured into the gel casting platform. Electrophoresis of the prepared samples was performed on a contour-clamped homogeneous electric field CHEF DR III system (Bio-Rad Laboratories, USA) with 2.5 liters of 0.5X TBE running buffer (Tris-borate-EDTA pH 8.0). Conditions were: initial switch time 2.2 seconds; final switch time 35.0 seconds; running time 22 hours; 120° angle; gradient 6.0 V/cm; temperature 14°C. After electrophoresis, the gel was stained for 1 hour in 250 milliliters of sterile solution containing 1 mg of ethidium bromide. The gel was photographed with Gel Documentation (SynGene, USA). A standard molecular weight marker (Bio-Rad Laboratories, USA) consisting of concatemers of the λ phage (lambda ladder) starting at 48.5 kb was used.

Results

Fingerprints of leptospiral reference serovars given by NotI restriction enzyme

Fingerprints for 28 reference serovar strains generated with restriction enzyme *Not*I resulted in 27 different patterns designated as PFGE patterns 1-27 (**Figure 1**) with the exception for serovars Copenhageni (lane 14) and Icterohaemorrhagiae (lane 15). The fingerprints by PFGE with discrimination power of 0.99 were reproducible and showed marked heterogeneity between member serovars. These 27 fingerprints are described as PFGE patterns 1 (serovar Australis), 2 (serovar Bangkok), 3 (serovar Bratislava), 4 (serovar Autumnalis), 5 (serovar New), 6 (serovar Rachamati), 7 (serovar Ballum), 8 (serovar Bataviae), 9 (serovar Canicola), 10 (serovar Cellidoni), 11 (serovar Djasiman), 12 (serovar Grippotyphosa), 13 (serovar Hebdomadis), 14 (serovar Copenhageni and Icterohaemorrhagiae), 15 (serovar Javanica), 16 (serovar Saigon), 17 (serovar Panama), 18 (serovar Pomona), 19 (serovar Pyrogenes), 20 (serovar Zanoni), 21 (serovar Ranarum), 22 (serovar Sarmin), 23 (serovar Hardjo), 24 (serovar Sejroe), 25 (serovar Wolffi), 26 (serovar Tarassovi), and 27 (serovar Patoc).

A restriction enzyme digestion of these 28 reference serovars DNA with *Not*I produced from 8 to more than 17 bands. Sizes ranged from less than 48.5 kb to 533.5 kb. No common band was found among these 28 strains. Moreover, serovars in the same serogroup showed distinct fingerprints. Serogroup Australis (serovars Australis, Bangkok, and Bratislava) produced PFGE patterns 1, 2, and 3, respectively. Serogroup Autumnalis (serovars Autumnalis, New, and Rachamati) produced PFGE patterns 4, 5, and 6, respectively. Serogroup Sejroe (serovars Hardjo, Sejroe, and Wolffi) produced PFGE patterns 23, 24, and 25, respectively.

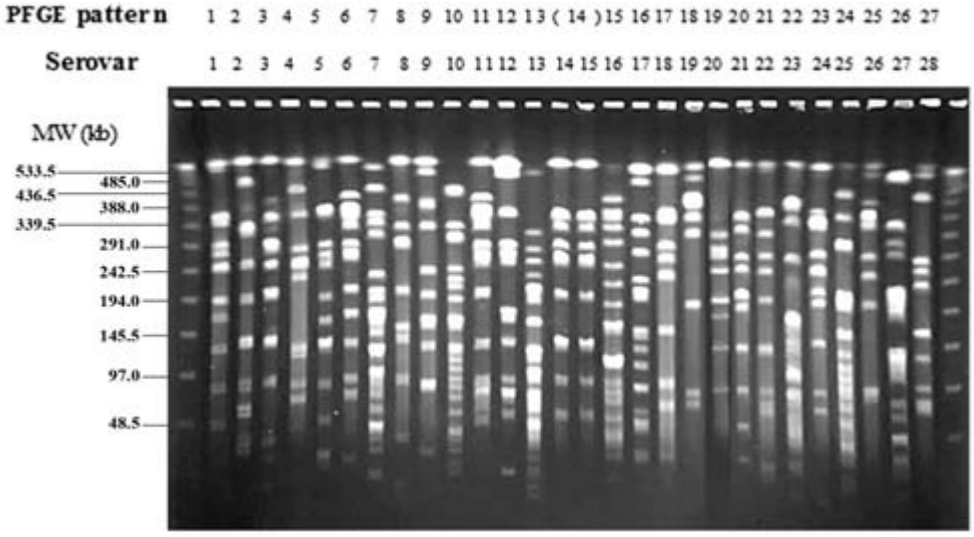

Figure 1. PFGE fingerprints of *Not*I digested chromosomal DNA of 28 reference *Leptospira* serovars strains. Lane M: 1 kb plus lambda ladder; lanes 1 to 3: serogroup Australis (serovars Australis, Bangkok, and Bratislava); lanes 4 to 6: serogroup Autumnalis (serovars Autumnalis, New, and Rachamati); lanes 7 to 13: serovars Ballum, Bataviae, Canicola, Cellidoni, Djasiman, Grippotyphosa, and Hebdomadis; lanes 14 to 15: serogroup Icterohaemorrhagiae (serovars Copenhageni and Icterohaemorrhagiae); lanes 16 to 19; serovars Javanica, Saigon, Panama, and Pomona; lanes 20 to 21: Serogroup Pyrogenes (serovars Pyrogenes and Zanoni), lanes 22 to 23: serovars Ranarum and Sarmin; lanes 24 to 26: serogroup Sejroe (serovars Hardjo, Sejroe, and Wolffi); lanes 27 to 28: serovars Tarassovi, and Patoc, respectively. Fragment sizes (in kilobase pairs) are indicated on the left.

Fingerprints of serovars Copenhageni and Icterohaemorrhagiae given by FspAI and SdaI restriction enzyme

Fingerprints after digestion with the enzymes *Fsp*AI and *Sda*I confirmed that both of serovars Copenhageni and Icterohaemorrhagiae could not be differentiated from each others (**Figure 2**). After restriction digestions of chromosomal DNA of the two serovars with either enzyme *Not*I, *Fsp*AI, or *Sda*I, they produced fingerprints with sizes range 48.5 kb to 533.5 kb and were the same pattern of the same enzyme. The same numbers of 9, 14, and 15 bands with the same sizes yielded from the enzymes *Not*I, *Fsp*AI, and *Sda*I, respectively. These results demonstrated similarities between the serovars Copenhageni and Icterohaemorrhagiae identified by using serotyping and genotyping.

Comparative fingerprints from the same serovars between field rat isolates and reference strains

PFGE fingerprints from four representative serovars as Pyrogenes, Bataviae, Autumnalis, and Australis of leptospires isolated from field rats, were compared with those from the corresponding reference strains. If two isolates had 100% band matching or only one band difference, these isolates were classified in the same group. Serovar Pyrogenes fingerprints from two rat isolates were similar to reference serovar Pyrogenes strain Salinem (**Figure 3A**). Seven rat isolates of serovar Bataviae, gave similar patterns with reference serovar Bataviae as shown in **Figure 3B**. Interestingly, fingerprints from two rat isolates of serovar Autumnalis did not match the reference serovar Autumnalis strain Akiyami A used in this study (**Figure 4A**). Moreover, the two rat isolates of serovar Australis presented different patterns from the reference serovar Australis strain Ballico used in this study (**Figure 4B**).

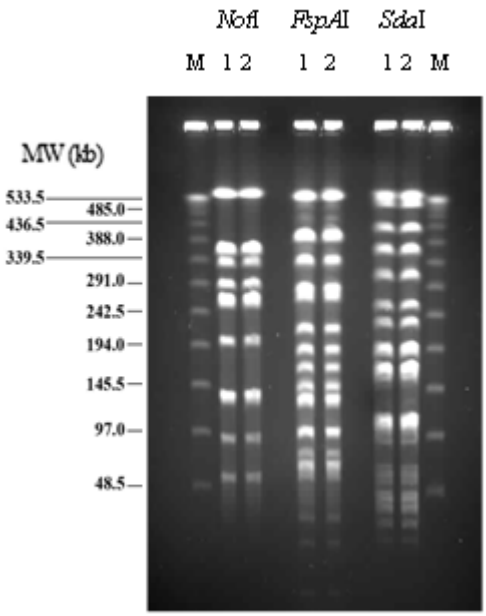

Figure 2. PFGE fingerprints of *Not*I, *Fsp*AI and *Sda*I digested chromosomal DNA of serovars Copenhageni and Icterohaemorrhagiae. Lane M: lambda marker; lanes 1 and 2: serovars Copenhageni and Icterohaemorrhagiae. Fragment sizes (in kilobase pairs) are indicated on the left.

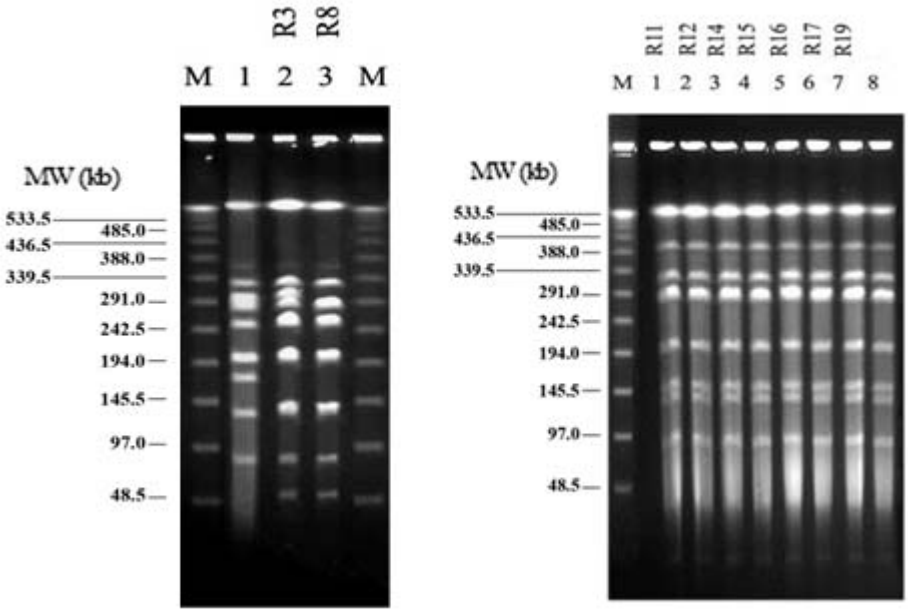

Figure 3. Similar PFGE fingerprinting patterns for the representative serovars Pyrogenes (**A**) and Bataviae (**B**) compared with those for corresponding reference serovar strains. **A:** Lane M: lambda marker; lanes 1-3: reference serovar Pyrogenes strain Salinem, representative serovar Pyrogenes from field rat isolates Nos. R3 and R8. **B:** Lane M: lambda marker; lanes 1-8: representative serovar Bataviae from field rat isolates Nos. R11, R12, R14, R15, R15, R16, R17, R19, and reference serovar Bataviae, respectively. Fragment sizes (in kilobase pairs) are indicated on the left.

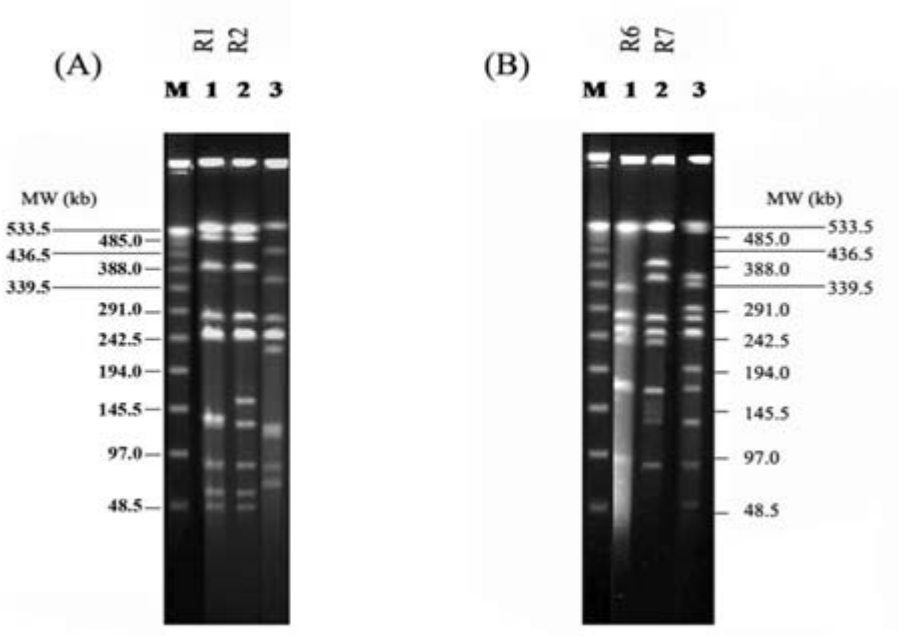

Figure 4. Different PFGE fingerprinting patterns for the representative serovars Autumnalis (**A**) and Australis (**B**) compared with those for corresponding reference serovar strains. **A**: Lane M: lambda marker; lanes 1-3: representative serovar Autumnalis from field rat isolates Nos. R1 and R2, and reference serovar autumnalis strain Akiyami A. **B**: Lane M: lambda marker; lanes 1-3: representative serovar Australis from field rat isolates Nos. R6 and R7, and reference serovar Australis strain Ballico. Fragment sizes (in kilobase pairs) are indicated on the left.

Discussion

Serovar identification is necessary for epidemiological surveillance since some serovars are known to have preferential animal reservoirs. For example, serovar Icterohaemorrhagiae is found in rats and serovar Canicola is found in dogs [2]. Some serovars are associated with certain clinical forms, such as serovars Icterohaemorrhagiae and Grippotyphosa, which were associated with Icterogenic Syndrome and Benign Meningitis Syndrome, respectively [2]. Investigation of serovars is not only useful for epidemiological studies, but also provides a strategy for prevention of the disease.

In this study, PFGE was performed to characterize 28 reference serovar strains of *Leptospira* spp. *Not*I was selected for PFGE analysis since it produced approximately 12 to 13 bands by using a genome sequence of *L. interrogans* serovar Lai strain 5660 as a template (http://insilico.ehu.es/digest/index.php?mo=Leptospira). In addition, *Not*I has proven to be a useful endonuclease for characterization among leptospires at serovar level reported by previous studies [9, 14].

PFGE successfully differentiated among reference strains. Each of 28 reference serovars possessed a unique profile, except serovars Copenhageni and Icterohaemorrhagiae, which yielded the same patterns. *Not*I fingerprint patterns of these reference strains produced patterns ranging from eight to more than 17 fragments. Each pattern was different from the others. The possibility of banding pattern heterogeneity may result from genetic rearrangement, such as translocation, inversions, or distribution of mobile genetic elements. For example, insertion sequences were found to scatter in the chromosomal DNA of serovars Lai and Copenhageni [17]. PFGE could not discriminate between serovars Copenhageni and Icterohaemorrhagiae. Additionally, these two serovars could not be distinguished by using *Fsp*AI and *Sda*I as seen in this study. The inability to differentiate serovars Copenhageni and Icterohaemorrhagiae, which are closely related, is consistent with previous studies using REA analysis with 15 endonucleases [18], *Eco*RI for digestion with 16S and 23S rRNA from *E. coli* as probes [12], PFGE of *Not*I digestion [19], AP-PCR fingerprints [20], MLVA assay [21], and 16S ribotyping of *Eco*RV and *Hind*III digestions [13]. These results indicate that seovars Copenhageni and Icterohaemorrhagiae are closely related, both serologically and genetically.

PFGE fingerprints from four representative field rat isolates as serovars Pyrogenes, Bataviae, Autumnalis, and Australis, were compared with those from the corresponding reference strains. The fingerprints from rat isolates as serovars Pyrogenes and Bataviae, were similar to reference serovar Pyrogenes strain Salinem and serovar Bataviae. The fingerprints from representative isolates for serovars Autumnalis and Australis did not match to their corresponding reference strains. As described, isolate of serovar Autumnalis yielded distinct patterns from reference serovar Autumnalis strain Akiyami A. Similarly, isolate of serovar Australis and reference serovar Australis strain Ballico showed different patterns. The results from serovars Autumnalis (not strain Akiyami A) and Australis (not strain Ballico) are consistent to those identified by 16S ribotyping of *Eco*RV and *Hind*III digestions as previously described [13]. Additionally, the findings were supported that phenotypes of serovar Hardjo strain Hardjobovis and strain Hardjoprajitno, were similar but genotyping of both strains were different as demonstrated by ribotyping, AP-PCR fingerprinting, and mapped restriction site polymorphisms in *rrs* and *rrl* genes [22]. These findings indicate that PFGE can determine new strains/serovars by demonstrating different fingerprint patterns from the reference serovar strains.

In the present study, PFGE showed high reproducibility and a high degree of discriminatory power to differentiate reference leptospires serovars. This technique had the ability to characterize leptospires isolated from field rats. It is a useful tool for characterization of leptospires in epidemiological studies.

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