Original Article

Optimization of Pulse-Field Gel Electrophoresis for *Borrelia burgdorferi* Subtyping*

GENG Zhen[1,2,+], HOU Xue Xia[1,+], HAO Qin[1,+], ZHOU Hai Jian[1], WANG Feng[2,#], and WAN Kang Lin[1,#]

1.State Key Laboratory for Communicable Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; 2. Department of Infectious Disease, The First Hospital, Jilin University, Changchun 130021, Jilin, China

Abstract

Objective To optimize the performance of Pulsed-Field Gel Electrophoresis (PFGE) for the comparison of inter-laboratory results and information exchange of *Borrelia burgdorferi* subtyping.

Methods A panel of 34 strains of *B. burgdorferi* were used to optimize PFGE for subtyping. In order to optimize the electrophoretic parameters (EPs), all 34 strains of *B. burgdorferi* were analyzed using four EPs, yielding different Simpson diversity index (D) values and the epidemiological concordance was also evaluated.

Results The EP of a switch time of 1 s to 25 s for 13 h and 1 s to 10 s for 6 h produced the highest D value and was declared to be optimal for *Mlu*I and *Sma*I PFGE of *B. burgdorferi*. *Mlu*I and *Sma*I were selected as the first and second restriction enzymes for PFGE subtyping of *B. burgdorferi* according to discrimination and consistency with epidemiological data.

Conclusion PFGE can be used as a valuable test for routine genospecies identification of *B. burgdorferi*.

Key words: Molecular subtyping; Pulse-Field Gel Electrophoresis; *Borrelia burgdorferi*

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INTRODUCTION

The spirochete *Borrelia burgdorferi*, the causative agent of the multi-system tick-borne infection, Lyme disease[1-2], is transmitted to humans primarily via known tick-bite[3-4]. It was not until early 1980s that Willy Burgdorfer discovered with colleagues a previously unidentified spirochetal bacterium, called *B. burgdorferi*, which was isolated and cultured from the midgut of *Ixodes* ticks, and subsequently from a variety of tick vectors and vertebrate hosts, including humans[1,5].

B. burgdorferi resembles most other spirochetes that it is a highly specialized, motile, two-membrane, spiral-shaped bacterium that exists primarily as an

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+GENG Zhen, HOU Xue Xia, and HAO Qin contributed equally to this study.

#Correspondence should be addressed to WAN Kang Lin, Tel: 86-10-58900776, Fax: 86-10-58900779, E-mail: wankanglin@icdc.cn; and WANG Feng, Tel: 86-431-88782732, Fax: 86-431-88782732, E-mail: wangfeng1234cn@yahoo.com.cn

Biographical notes of the first authors: GENG Zhen, male, born in 1976, M.D, assistant researcher, major in Lyme disease; co-first author: HOU Xue Xia, female, born in 1968, associate technician, major in Lyme disease; HAO Qin, female, born in 1975, Ph.D., major in Lyme disease.

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extracellular pathogen. It is fastidious and difficult to culture *B. burgdorferi in vitro* which requires a special enriched medium and low oxygen tension. At least 11 genospecies of *B. burgdorferi* have been identified through a variety of methods, such as DNA-DNA hybridization (DDH) and 16S rRNA analysis[4-5]. Some of the genospecies such as *B. burgdorferi sensu strict* (*B. burgdorferi ss*), *B. garinii, and B. afzelii*, have been shown to cause Lyme disease in humans, whereas the pathogenic status of other genospecies remains unknown. In addition, there is considerable variation in disease presentation among the Borrelia genotypes. For example, *B. burgdorferi ss, B. garinii, and B. afzelii* are predominantly and respectively associated with arthritis, neurological problems, and skin problems[6-7]. It is therefore critical from a public health perspective that scientists have reliable molecular methods in order to identify different genospecies of *B. burgdorferi*.

Preliminary reports have demonstrated that Pulsed-Field Gel Electrophoresis (PFGE) is a useful and an important molecular level test for subtyping of many pathogenic bacteria[8-11]. PFGE separates large DNA molecules by using an electric field that periodically changes direction to a gel matrix, and makes use of restriction enzymes to create distinct patterns of restriction enzymes. The number and size of the restriction fragments depends on the restriction enzymes, while the band distribution in the gel depends on the electrophoretic parameters (EPs). To date, several different PFGE protocols have been used to characterize *B. burgdorferi*[5,12-15]. In order to facilitate the comparison among different studies on *B. burgdorferi*, it is critical to standardize PFGE protocols. We therefore used in this study the standard protocol for *Salmonella* plug preparation to standardize the bacterium concentration, select appropriate restriction enzymes and optimize EPs.

MATERIALS AND METHODS

Bacterium Strains and Culture Conditions

A panel of 34 *B. burgdorferi* strains was collected and used for the development of the PFGE protocol as described below. Five strains were obtained from the American Type Culture Collection (ATCC): B31 (item number 35 210), 51 383 (item number 51 383), Fuji (item number 51 991), 700 557 (item number 700 557), and 51 992 (item number 51 992). Strains 20 047, VS461, ASF, and NT31 were provided by R. C. Johnson and T. J. Quan[16]. The remaining 25 strains were collected in China from rats, ticks or patients' blood or cerebrospinal fluid, and the epidemiological details are listed in Table 1.

Table 1. Basic Information and Genospecies Identification of 34 Strains Used for this Study

Strain	Time of Isolation	Origin	Geographical Location	Species			
				PFGE (*Mlu*I)	PFGE (*Sma*I)	RFLP[c]	MLSA[c]
CS4	September, 1992	Rabbit bladder	Hunan Province, China	I[d]	I[d]	*B. burgdorferi sensu stricto*	1[e]
B31[a]		*Ixodes dammini*	New York, USA	I	I	*B. burgdorferi sensu stricto*	1
20047	August, 1993	*Ixodes ricinus*	France	II[d]	II[d]	*B. garinii*	2[e]
PD91	August, 1991	human blood	Inner Mongolia Autonomous Region, China	II	II	*B. garinii*	2
MD21	September, 1997	Rattus norvegicus	Guangdong Province, China	II	II	*B. garinii*	2
SF3	November, 1987	*Ixodes persulcatus*	Heilongjiang Province, China	II	II	*B. garinii*	2
Y3	August, 1993	human blood	Heilongjiang Province, China	II	II	*B. garinii*	b
XI91-12	June, 1991	*Ixodes persulcatus*	Xinjiang Uygur Autonomous Region, China	II	II	*B. garinii*	2

Continued

Strain	Time of Isolation	Origin	Geographical Location	Species			
				PFGE (*Mlu*I)	PFGE (*Sma*I)	RFLP[c]	MLSA[c]
JL13	July, 2004	*Ixodes persulcatus*	Jilin Province, China	II	II	*B. garinii*	2
JP2	June, 1992	*Ixodes persulcatus*	Jilin Province, China	II	II	*B. garinii*	2
JP17	June, 1992	*Ixodes persulcatus*	Jilin Province, China	II	II	*B. garinii*	2
JP25	June, 1992	*Ixodes persulcatus*	Jilin Province, China	II	II	*B. garinii*	2
JC2-10	June, 2006	*Ixodes persulcatus*	Jilin Province, China	II	II	*B. garinii*	2
JC2-14	June, 2006	*Ixodes persulcatus*	Jilin Province, China	II	II	*B. garinii*	2
NT31	October, 1995	*Ixodes persulcatus*	Hokkaido, Japan	II	II	*B. garinii*	2
ASF	October, 1995	Apodemus agrarius	Hokkaido, Japan	II	II	*B. garinii*	2
51383[a]		*Ixodes ricinus*	France	II	II	*B. garinii*[a]	
Fuji[a]		*Ixodes persulatus*	Fuji, Japan	II	II	*B. garinii*[a]	
VS461	August, 1993	human csf	Switzerland	III[d]	III[d]	*B. afzelii*	3[e]
FP1	June, 1991	human blood	Sichuan Province, China	III	III	*B. afzelii*	3
R9	September, 1989	human blood	Heilongjiang Province, China	III	III	*B. afzelii*	3
LIP94-11	October, 1994	*Ixodes persulcatus*	Liaoning Province, China	III	III	*B. afzelii*	3
LIP94-4	October, 1994	*Ixodes persulcatus*	Liaoning Province, China	III	III	*B. afzelii*	B
LPR30	August, 1996	Rattus coxingi	Guangdong Province, China	III	III	*B. afzelii*	3
SZ21	May, 1992	*Haemaphysalis longicornis*	Beijing Municipality, China	III	III	*B. afzelii*	3
GM4	March, 1998	Apodemus agrarius	Guizhou Province, China	III	III	*B. afzelii*	3
51992[a]		human skin	Germany	III	III	*B. afzelii*[a]	
GL18	October, 2001	Apodemus agrarius	Guizhou Province, China	IV[d]	IV[d]	*B. valaisiana*	4[e]
QTDS2	October, 2006	*Ixodes granulatus*	Guizhou Province, China	IV	III	*B. valaisiana*	4
QX-S13	October, 2006	*Ixodes granulatus*	Guizhou Province, China	IV	IV	*B. valaisiana*	4
QLYZSP1	October, 2006	*Ixodes granulatus*	Guizhou Province, China	IV	IV	*B. valaisiana*	4
GDsh1	June, 2010	Rattus norvegicus	Guangdong Province, China	IV	IV	*B. valaisiana*	B
JX1	September, 2010	Apodemus agrarius	Jiangxi Province, China	IV	IV	*B. valaisiana*	b
700557[a]		*Ixodes dentatus*	Millbrook, USA	V[d]	IV	*B. andersonii*[a]	

Note. [a], Strain bought from ATCC, of which the species was noted in the directions of strain. [b], Strains were not analyzed for genotyping with this method. [c], See references: 16, 24, 25 and 26. [d], I to V - five genospecies including *B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, *B. valaisiana*, and *B .andersonii* typed by PFGE. [e], 1 to 5 - five genospecies including *B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, *B. valaisiana*, and *B .andersonii* typed by MLSA.

All strains were inoculated into Barbour-Stoenner-Kelly II (BSKII) medium (Statens serum institut, Denmark) and cultured at 33 °C for 4 to 7 days for PFGE plug preparation. Three strains (20 047, 51 992, B31) were analyzed in a pilot study to select the standardized bacterium concentrations, and another three strains (PD91, GDsh1, JX1) were analyzed randomly for selection of restriction endonucleases due to that all strains of *B. burgdorferi* share similar growing characterization. Furthermore, 11 strains were also selected randomly for investigation of protocol reproducibility.

Enzyme Selection

Enzymes for the pilot study were selected based on the complete *B. burgdorferi* genomic sequence (NC 001318, NC 011728) published in the GenBank, using DNASTAR 5.01 software (DNASTAR, Inc., WI, 136USA). Reports on *B. burgdorferi* PFGE analysis indicated that *Sma*I and *Mlu*I are the commonly used enzymes in these kind of studies[12,14]. The pilot study was conducted with three strains (PD91, GDsh1 and JX1) and the optimal enzyme was selected based on the distribution of bands. EP: 1 s to 25 s for 19 h.

PFGE Protocol

The PFGE protocol used in this study was based on the PulseNet 1-day standardized PFGE protocol for *Vibrio cholerae*[17]. *B. burgdorferi* were harvested by centrifugation at 12 000 rpm (19 117×g) for 30 min, the pellet was then washed twice in 0.01 mol/L pH7.4 phosphate-buffered saline (PBS) and was finally resuspended in a polystyrene tube (Falcon; 12 mm×75 mm) with sterile PBS. The suspension was adjusted to optical density values of 3.0 (9×10^8 CFU/mL), 4.0 (12×10^8 CFU/mL), 5.0 (15×10^8 CFU/mL), 6.0 (18×10^8 CFU/mL), and 7.0 (21×10^8 CFU/mL) using a portable photometer (bioMérieux Densimat). *Salmonella* Serotype Braenderup 9812 was used as a DNA size marker as recommended by the PulseNet[18].

The resulted *B. burgdorferi* gel slices were digested with 10 U *Sma*I/slice (TakaraBio Dalian, China) or with a corresponding amount of other enzymes at 30 °C or 37 °C for 2 h. H9812 was digested with 30 U *Xba*I/slice (TakaraBio Dalian, China) at 37 °C for 2 h. Electrophoresis was then performed using a CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA). The Images were captured on a Gel Doc 2000 system (Bio-Rad) and converted to TIFF for computer analysis. Test strain and H9812 plugs were prepared and digested in parallel. All electrophoresis procedures were performed with a voltage gradient of 6 v/cm, an included angle of 120° and a linear ramp.

Computer Analysis of PFGE Patterns

Patterns produced by PFGE were analyzed using the BioNumerics software package (version 5.1). Clustering was created with the unweighted-pair group method using average linkages (UPGMA). Fragments of less than 20.5 kb were not analyzed in this study.

Optimization of Electrophoretic Parameters

Thirty four strains were analyzed following *Sma*I and *Mlu*I digestion using four EPs (EP-a, EP-b, EP-c, and EP-d) (Table 2 and Table 3). EP-a was recommended for use with the CHEF Mapper system, based on the size of restriction fragments and was modified to produce a distinct band pattern, while EP-b, EP-c, and EP-d were selected from a pilot study. The Simpson diversity index (D value)[19-20] was applied to compare the discriminatory powers of each parameter. Accordingly, EP producing high D values is optimal for distinguishing strains[21].

Table 2. Features of Four Electrophoretic Parameters with *Sma*I Digestion

EP	Switch Time (s)	Total Run Time (h)	D Value
EP-a	1-25	19	0.9661
EP-b	1-25 1-10	13 6	0.9893
EP-c	1-25 1-10	6 12	0.9404
EP-d	1-10 20-25	13 6	0.9422

Table 3. Features of Four Electrophoretic Parameters with *Mlu*I Digestion

EP	Switch Time (s)	Total Run Time (h)	D Value
EP-a	1-25	19	0.9910
EP-b	1-15	18	0.9893
EP-c	1-25 1-10	13 6	0.9910
EP-d	1-10 20-25	13 6	0.9910

Evaluation of PFGE Protocols, Focusing on Typeability, Reproducibility, and Epidemiological Concordance

Based on the methods described by Struelens[22], typeability was calculated as the percentage of distinct bacterial strains that could be assigned a specific pattern. Reproducibility was evaluated by repeat analysis of 11 strains, which were analyzed on three separate CHEF Mapper systems followed by clustering of the patterns obtained on independent runs. Epidemiological concordance was also evaluated in this study by analyzing 34 strains of *B. burgdorferi*.

RESULTS

Selection of Enzymes

A theoretical enzyme selection using DNASAR 5.01 software, was expanded (0-100) to include all suitable enzymes. As the result, the following enzymes were selected for use in the pilot study: *Sma*I, *EcoR*I, *Hha*I, *Cla*I, *Mlu*I, *Xho*I, and *Apa*I. The selection was based on the requirement for generation of a low number of relatively small fragments and the consideration of cost effectiveness. The Imaging data showed that the majority of bands produced following digestion with *Eco*RI, *Hha*I, *Cla*I, *Xho*I, and *Apa*I could be resolved using this method (Figure 1). Therefore, the pattern produced following digestion with *Sma*I and *Mlu*I was selected as optimal one for the purposes of PFGE in this study.

Optimization of Electrophoretic Parameters of SmaI and MluI Digestion

Data obtained from this study demonstrated that a turbidity concentration of *B. burgdorferi* exceeding 4.0 (12×10^8 CFU/mL) was needed for clear imaging (Figure 2). EP-a (switch time of 1 s to 25 s for 19 h) was indicated by the CHEF Mapper system as the optimal condition for *Sma*I and *Mlu*I digestion of *B. burgdorferi* based on the size of restriction fragments. However, these EP did not allow sufficient resolution of bands to distinguish fragments of less than 200 kb. Therefore, EP-a was modified in order to improve resolution (EP-b, EP-c, EP-d) for *Sma*I and *Mlu*I digestion. Comparison of all four EPs following *Sma*I digestion produced D values of 0.9661, 0.9893, 0.9404, and 0.9422 respectively (Table 2). Based on the principle that high D values are optimal for distinguishing strains and are considered to be the standard for evaluating the discriminatory power of other enzymes[19,21], the D value 0.9893 of EP-b was selected as the optimal parameter for *Sma*I digestion.

Comparison of all four EPs following *Mlu*I digestion produced D values of 0.991, 0.9893, 0.991, and 0.991 respectively (Table 3). The D value 0.991 of EP-c was then selected as the optimal parameter for *Mlu*I, with the same switch time of 1 s to 25 s for 13 h and 1 s to 10 s for 6 h used for *Mlu*I and *Sma*I.

The results shown in Figure 3 and Figure 4 (See the website of this journal) demonstrated that *B. garinii* strains JL13 and NT31 always clustered within the *B. valaisiana* genospecies with *Sma*I digestion, but JL13 and NT31 could cluster within the *B. garinii*

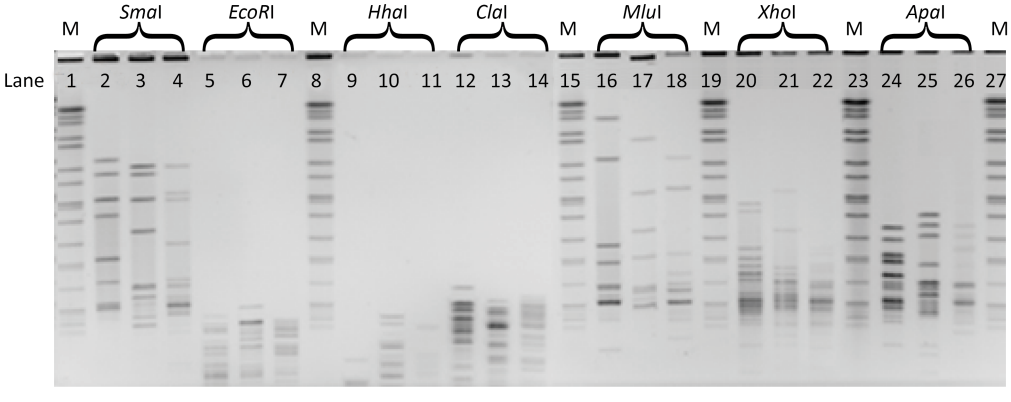

Figure 1. PFGE images of three *B. burgdorferi* strains digested with restriction enzymes. Lanes 1, 8, 15, 19, 23, and 27: size standards (M). *Sma*I (lanes 2 to 4), *EcoR*I (lanes 5 to 7), *Hha*I (lanes 9 to 11), *Cla*I (lanes 12 to 14), *Mlu*I (lanes 16 to 18), *Xho*I (lanes 20 to 22), *Apa*I (lanes 24 to 26). PD91 (lanes 2, 5, 9, 12, 16, 20, and 24); GDsh1 (lanes 3, 6, 10, 13, 17, 21, and 25); JX1 (lanes 4, 7, 11, 14, 18, 22, and 26).

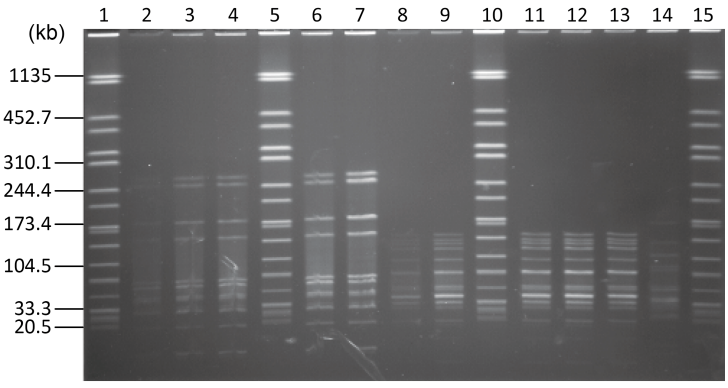

Figure 2. PFGE of *Sma*I restriction fragments of *B. burgdorferi* DNA for selection of concentration of three standard strains.Lane 1, 5, 10, and 15 are H9812 (molecular weight); Lane 2, 3, 4, 6, and 7 are 20047, Lane 8, 9, 11, 12, and 13 are 51992. Bacterium concentrations yield optical density values of 3.0, 4.0, 5.0, 6.0, and 7.0 respectively. Lane 14 is B31. Bacterium concentrations yield an optical density of 4.0. EP: 6.8 s to 38.4 s for 19 h.

genospecies with *Mlu*I digestion. And *B. valaisiana* strain QTDS2 clustered within the *B. afzelii* genospecies with *Sma*I digestion, and *B. andersonii* strain 700557 clustered within the *B. valaisiana* genospecies with *Sma*I digestion, but QTDS2 and 700557 could cluster within the correct genospecies with *Mlu*I digestion.

Typeability, Reproducibility, and Epidemiologic Concordance

PFGE with *Sma*I digestion allowed subtyping of all *B. burgdorferi* strains and achieved the same level of typeability (100%) as that achieved with *Mlu*I digestion.

Eleven strains were analyzed on three separate occasions using EP-b for *Sma*I PFGE. The patterns produced from same strains were shown to be indistinguishable across independent experiments, thus demonstrating good reproducibility of EP-b. Similar reproducibility was demonstrated using EP-c for *Mlu*I PFGE (data not shown).

PFGE data from all 34 strains were evaluated for the concordance with data obtained from epidemiological studies using two alternative molecular typing methods (Table 1). All 34 strains were divided into five genospecies (I, II, III, IV, and V) by *Mlu*I PFGE and correlated with strains *B. burgdorferi ss*, *B. garinii*, *B. afzelii*, *B. valaisiana,* and *B. andersonii* respectively by restriction fragment length polymorphism (RFLP) and multilocus sequence analysis (MLSA).

DISSCUSSION

PFGE is considered to be the “gold standard” technique for molecular typing due to its high degree of reproducibility and unprecedented resolving power[21]. We can say that PFGE is better for typing than PCR. It can reflect the more bacterium genetic information than PCR typing because the bacterium whole genomic sequencing is used.

Some previous studies have investigated the influencing factors to PFGE results, including plug preparation, cell lysis, enzymatic digestion and electrophoretic parameters[5,13]. EP, including switch times and total run time, influence the distribution of restriction fragments in the electrophoretic gel, and further influence the discriminatory power of this method. Yet, it is still necessary to establish a practical approach for the identification of optimal EP.

In this research, the selection of optimal EP with the highest discriminatory power for PFGE subtyping of *B. burgdorferi* was established in a pilot study and the protocol was optimized afterward. It is anticipated to be used widely for *B. burgdorferi* genotyping in epidemiological investigations, surveillance and basic research.

Restriction enzymes produce distinct banding patterns and often different band numbers[23]. High band numbers are known to reduce the clarity of the image[20]. The principle underlying selection of the restriction enzyme for PFGE is based on the production of distinct patterns with uniformly distributed bands. According to the findings from both software analysis and a pilot study, *Sma*I and *Mlu*I were finally selected as the restriction enzymes in this study.

The concentration of bacteria is critical for

distinct imaging of PFGE results. Pilot studies revealed that concentrations producing turbidity values greater than 4.0 (12×10^8 CFU/mL) yielded clear images. This parameter was subsequently used for PFGE analysis of 34 strains of *B. burgdorferi* and the results suggested that this concentration of *B. burgdorferi* was suitable for *Sma*I or other enzymes, such as *Mlu*I, which yield similar band numbers.

EP producing high D values, which increase the level of discriminatory power of PFGE, are considered to be optimal for distinguishing two bacterial strains[19-21]. For a group of strains, the electrophoretic parameter producing the highest D value is considered to be optimal. Strains selected for analysis were obtained from varying geographic locations at different times in order to reflect the traits of common *B. burgdorferi* PFGE patterns.

A genetic relationship dendrogram was generated from the PFGE patterns after *Sma*I or *Mlu*I digestion of 34 *B. burgdorferi* strains using BioNumerics software. The majority of strains were classified within corresponding groups with homology scores of different genomes being under 40% (Figure 3 and Figure 4, see the website of this journal), indicating that different species of *B. burgdorferi* can be distinguished broadly using the PFGE technique, which is therefore valuable for the appllication in epidemiologic surveys and related research. These observations are consistent with others reports[12-15].

However, there were some problems encountered in the epidemiological investigation of *B. burgdorferi* by PFGE following single *Sma*I digestion, such as the *B. garinii* strains JL13 and NT31 always clustered within the *B. valaisiana* genospecies with *Sma*I digestion, but JL13 and NT31 could cluster within the *B. garinii* genospecies with *Mlu*I digestion, indicating that a combination of two restriction enzymes may increase the discriminatory power of this method. In the present study, *Mlu*I and *Sma*I PFGE produced D values of 99.10% and 98.93%, respectively, showing higher discriminatory power of *Mlu*I PFGE .

The epidemiological concordance of *Sma*I PFGE and *Mlu*I PFGE was also evaluated in the present study in a comparison with RFLP and MLSA analysis. The majority of the strains were subtyped as *B. burgdorferi ss*, *B. garinii*, *B. afzelii*, *B. valaisiana,* and *B. andersonii* by PCR-RFLP and *Sma*I PFGE with the exception of QTDS2 and 700557. *B. valaisiana* strain QTDS2 clustered within the *B. afzelii* genospecies with *Sma*I digestion, and *B. andersonii* strain 700557 clustered within the *B. valaisiana* genospecies with *Sma*I digestion, but QTDS2 and 700557 could cluster within the correct genospecies with *Mlu*I digestion. We can therefore conclude that *Mlu*I and *Sma*I were selected as the first and second restriction enzymes, respectively, for PFGE subtyping of *B. burgdorferi*. Previous results have shown that PFGE has a high degree of consistency with other molecular typing methods for *B. burgdorferi* subtyping, such as PCR-RFLP, ribotyping and ospA serotyping[12-14]. And in this study, PFGE exhibited a high degree of consistency with MLSA and PCR-RFLP for *B. burgdorferi* subtyping and a higher discriminatory power for subtyping of strains of other species.

In short, a panel of *B. burgdorferi* strains was studied using PFGE method based on the principle that there is a direct correlation between D values and the discriminatory power of this technique. Four different EP were investigated for optimization of PFGE with *Sma*I and *Mlu*I digestion in order to identify the experimental conditions for optimal discriminatory power. And the protocol established in the present study confirmed that *Mlu*I and *Sma*I are suitable for use in *B. burgdorferi* genotyping by using identical EP (1 s to 25 s for 13 h and 1 s to 10 s for 6 h). These data demonstrated that *B. burgdorferi* identification by PFGE exhibited typeability, reproducibility and epidemiologic concordance with RFLP and MLSA and therefore, represents a valuable technique for further development and to be implemented as a method for routine genospecies identification of *B. burgdorferi*.

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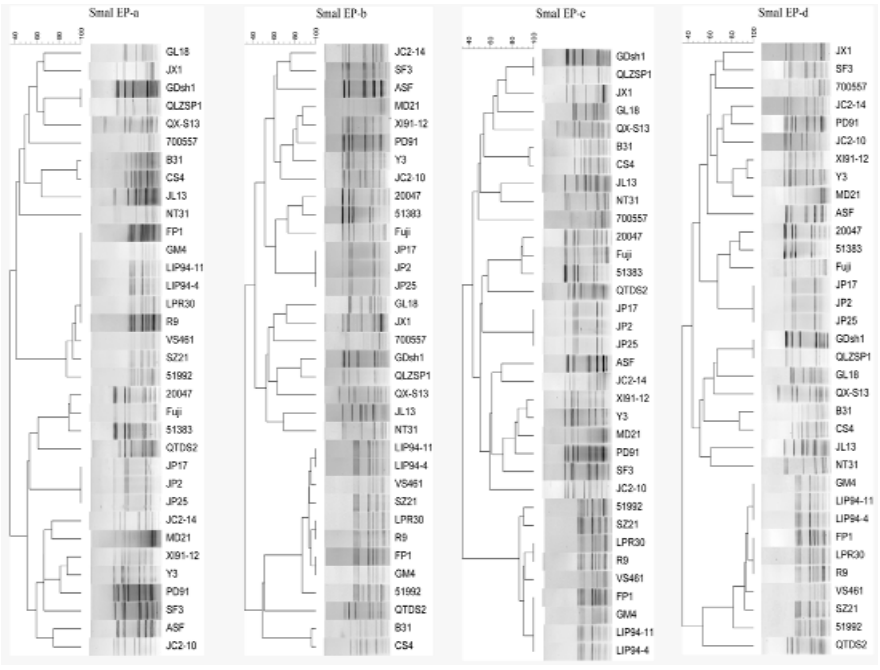

Figure 3. Genetic relationship dendrogram of 34 *B. burgdorferi* strains using PFGE patterns obtained after *Sma*I digestion using BioNumerics software. The tolerances were determined according to H9812 strain patterns. PFGE images were obtained using the EP-a, EP-b, EP-c and EP-d parameter cluster respectively

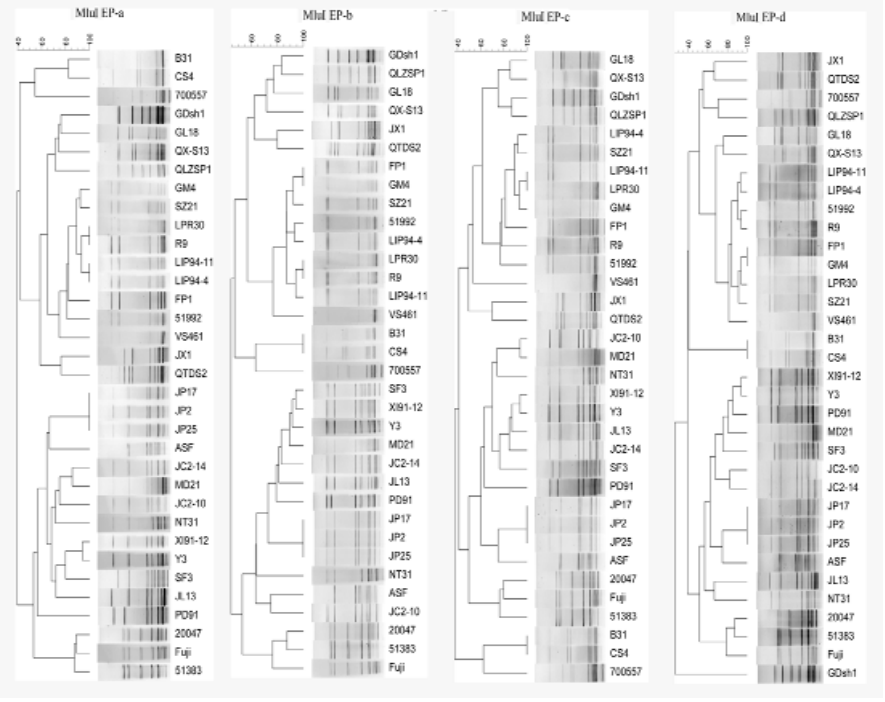

Figure 4. Genetic relationship dendrogram of 34 *B. burgdorferi* strains using PFGE patterns obtained after *Mlu*I digestion using BioNumerics software. The tolerances were determined according to H9812 strain patterns. PFGE images were obtained using the EP-a, EP-b, EP-c and EP-d parameter cluster respectively.