Molecular Typing of Leptospira Clinical Isolates in Thailand Using Multiple-locus Variable Number of Tandem Repeat Analysis (MLVA)

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Abstract

Leptospirosis is a re-emerging zoonosis of the tropical and subtropical areas of the world. The causative agent, *Leptospira* spp., can be classified into more than 300 serovars based on a serological technique and 15 genomospecies according to DNA-DNA hybridization. To date, the Microscopic Agglutination Test (MAT) is the only major means to identify *Leptospira* serovars for epidemiological tracing. MAT is a sophisticated test involving a large panel of serovar-specific antisera. The laboratory personnel is endangered by infection from the requirement of a large number of living leptospires. Therefore, the development of a new simpler technique is essential. To date, whole genome sequences of four *Leptospira* serovars have been released and over 60 variable number of tandem repeats (VNTR) with difference in length and copy numbers have been identified. Thus, the objective of this study is to evaluate the efficacy of multiple-locus of VNTR analysis (MLVA) in *Leptospira* serovars identification. A total of 30 *Leptospira* reference serovars and 55 *Leptospira* spp. isolated from patients with confirmed leptospirosis in Thailand during 2002 to 2006, were analyzed for the presence of five VNTR loci namely V27, V29, V30, V36 and V50 by PCR. A total of 38 MLVA patterns were identified in this study. Twenty-three MLVA patterns were identified from 30 reference samovars. Three serovars provided similar MLVA patterns and six serovars provided no amplification products. Some of the *Leptospira* patient isolates yielded identical MLVA patterns to that of the reference serovars, and they provided an extra 15 MLVA patterns. The MLVA results in this study completely conformed to the serovar identification from either MAT or latex agglutination. In conclusion, our data further emphasize the discrimination power of MLVA in *Leptospira* serovar identification. Nevertheless, more VNTR loci and a larger panel of reference serovars need to be investigated to obtain the complete Thai MLVA pattern database for all *Leptospira* serovars.

1. Introduction

Leptospirosis is a worldwide zoonosis caused by a spirochete belonging to the genus *Leptospira*. Human infections usually result from direct contact with urine and secretions of sick mammals, in particular rodents [1]. Soil and water that are contaminated with infected animal urine are also known as *Leptospira* vehicles [1]. As a consequence of the association of certain *Leptospira* serovars with disease severity and the great diversity in the species, a tool that is simple and can readily distinguish *Leptospira* serovars during outbreak or epidemiological tracing assessment is indeed an urgent need. To date, conventional diagnosis and serovar identification of *Leptospira* are mainly based on the serological method, *i.e.*, microagglutination test (MAT) [2]. MAT is a complicated and laborious test due to the requirement of a large number of serovar-specific antisera and maintenance of a panel of *Leptospira* serovars representative of at least 23 reference serogroups in culture, concomitantly [2, 3]. Although, serovar-specific monoclonal antibodies are also use for rapid diagnosis and serovar identification, their availability are very limited in term of quantity and serovar coverage [1].

Although molecular techniques such as PCR, restriction fragment length poly morphism (RFLP), and pulsed-field gel electrophoresis (PFGE) have been helpful in classifying leptospires at the serogroup level, these methods are time consuming and require expensive equipment and expertise [4, 5]. Variable Number of Tandem Repeats (VNTR) have been described previously in other organisms [6, 7]. VNTR are the repeated DNA sequences of varying copy number [6]. An ability to detect VNTRs in microorganisms requires whole genome sequences and software that can search for VNTR loci from those sequences [8]. Once the polymorphisms are located, flanking primers can then be designed to amplify these variable length regions. Thus the unique copy numbers in each series can be assessed using the size of the resultant amplicons [9, 10, 11]. Furthermore, when VNTR analysis is applied to the multiple loci as a typing scheme such as in multiple locus VNTR analysis (MLVA), greater discriminatory power and more accurate determination of genetic relatedness is achieved [8, 9, 10].

In this study, we employed five leptospira specific-VNTR loci for testing their efficacy in serovar identification of *Leptospira* strains isolated from Thai patients.

2. Materials and methods

2.1. *Leptospira* spp. strains

Pathogenic and non-pathogenic *Leptospira* spp. strains belonging to eight genomospecies, *i.e. L. borgpetersenii, L. interrogans, L. kirschneri, L noguchii, L .meyeri, L. weillii,* and *L. biflexa* were used as reference strains in this study (Table 1). They were provided by the Department of Medical Sciences, Ministry of Public Health, Thailand, and the Wellcome Unit, faculty of Tropical Medicine, Mahidol University, Bangkok. Fifty-five *L. interrogans* strains isolated from the blood of patients with confirmed leptospirosis cases at Maharaj Hospital, Nakorn Ratchasima Province, and north-eastern Thailand were studied for the presence of five VNTR loci and their size. These isolates were previously demonstrated to be positive for 16S DNA and *wzy* genes of *Leptospira* spp. [13] (Table 2).

For genomic DNA preparations, all strains of *Leptospira* spp. were grown in liquid EMJH medium (Difco, Detroit, MI, USA) supplemented with 1% heat inactivated normal rabbit serum and 100 µg/ml 5-fluorouracil at 30ºC under aerobic conditions.

Table 1 *Leptospira* reference strains used in this study

Number	Serovars	Serogroup	Genome Species	Strain
1	Australis	Australis	*L. interrogans*	Ballico
2	Autumnalis	Autumnalis	*L. interrogans*	Akiyami
3	Ballum	Ballum	*L. borgpetersenii*	Mus 127
4	Bangkinang	Autumnalis	*L. interrogans*	Bangkinang 1
5	Bataviae	Bataviae	*L. interrogans*	Swart
6	Bratislava	Australis	*L. interrogans*	Jez Bratislava
7	Canicola	Canicola	*L. interrogans*	Hond Utrecht IV
8	Carlos	Autumnalis	*L. interrogans*	C3
9	Celledoni	Celledoni	*L. weilii*	Celledoni
10	Cynopteri	Cynopteri	*L. kirshneri*	3522C
11	Djasiman	Djasiman	*L. interrogans*	Djasiman
12	Fortbragg	Autumnalis	*L. noguchii*	Fort Bragg
13	Grippotyphosa	Grippotyphosa	*L. kirshneri*	Moskva
14	Hebdomadis	Hebdomadis	*L. interrogans*	Hebdomadis
15	Icterohaemorrhagiae	Icterohaemorrhagiae	*L. interrogans*	RGA
16	Javanica	Javanica	*L. interrogans*	Veldrat Bat, 46
17	Mini	Mini	*L. borgpetersenii*	
18	Mujunkunmi	Autumnalis	Uncertain	Yezsh 273
19	Naam	Icterohemorrhagiae	*L. interrogans*	Naam
20	Nanla	Autumnalis	Uncertain	A6
21	New	Autumnalis	*L. interrogans*	Heusden P2062
22	Panama	Panama	*L. noguchii*	CZ214K
23	Pomona	Pomona	*L. interrogans*	Pomona
24	Pyrogenes	Pyrogenes	*L. interrogans*	Salinem
25	Ranarum	Ranarum	*L. meyeri*	Lowa City Frgo
26	Saigon	Louisiana	*L. noguchii*	L79
27	Sarmin	Sarmin	*L. weilli*	Sarmin
28	Sejroe	Sejore	*L. borgpetersenii*	M84
29	Shermani	Shermani	*L. santarosai*	LT821
30	Wolffii	Sejroe	*L. interrogans*	3705

Table 2 Fifty-five *Leptospira* patient isolates used in this study

Genome Species	Serovar identification by MAT	No. tested isolate
L. interrogans	Authumnalis	32
	Ballum	1
	Bataviae	2
	Bratislava	1
	Erincelaunti	1
	Weerasinghe	1
	Grippotyphosa	1
	Javanica	1
	Mendanensis	2
	Mini	1
	Hebdomadis	1
	Pyogenes	3
	Sejroe	3
	Saigon	1
	Shermani	1
L. wellii	Korat	3

2.2. DNA techniques

Genomic DNA was extracted from ca. 1×10^9 cells of each strain of *Leptospira* spp. grown to late log phase using Wizards genomic DNA purification kit (Promega, Madison, Wl., USA). The dye-terminator chemistry (Applied Biosystems, Carlsbad, CA., USA) and sequencing or custom-made oligonucleotide primers (Bio Basic Inc., Ontario, Canada) were used for automated DNA sequencer (model 3100; Applied Biosystems).

2.3. Polymerase chain reaction (PCR)

The presence of five VNTR loci namely V27, V29, V30, V36 and V50 were determined by PCR. DNA primer sequences used in the PCR and their respective references are shown in Table 3. Each PCR reaction mixture consisted of 2.5 µl 10× buffer (1.5 Mm $MgCl_2$, 0.2 mM deoxynucleotide triphosphate) (Fermentas, Ontario, Canada), 1 µM of individual primers (Bio Basic, Canada), 0.75 unit of Taq DNA polymerase (Fermentas) and 50 ng of genomic DNA. The final volume was adjusted to 25 µl by adding sterile ultra pure distilled water. The mixture was subjected to amplification in Thermocycler (Mastercycler *gradient*, Eppendorf, Hamburg, Germany). The steps and conditions of the PCR for DNA amplification of individual genes are given in Table 3. The PCR spliced products were subjected to 2% agarose (USB, Cleveland, OH, USA) gel electrophoresis, stained by 1% ethidium bromide (Sigma Chemical Co., St. Louis, MO, USA) and then visualized using Gel Documentation System (Ge1Doc 2000, Bio-Rad, Hercules, CA, USA)

Table 3 VNTR loci and primers used in this study

Primer Name	Sequence (5'-3')	Locus	Annealing Temperature	Amplification Cycles	Reference
V27F V27R	TCGTCGGGTGAGCTAAAATAT TTCTTTCGGTGGCAAGGTTT	V27	56ºC	30	[9]
V27NF V27NR	AAGTTCGTCGGGTGAGC TGATTTCTTTCGGTGGC	V27	60 ºC	30	[10]
V29F V29R	ATCGTTTTGGCAGTTTTTGCT CTAGAAAATTCCGCGTAGGG	V29	56ºC	30	[9]
V29NF V29NR	TGGGTGCCGGGTTGT ATGCCACATCTCATCCATTA	V29	60 ºC	30	[10]
V30F V30R	AAGTAAGATAGGTTCGGCGTTTA ACTTGGGTGTTAATCGCAAAA	V30	56ºC	30	[9]
V30NF V30NR	ATAGGTTCGGCGTTTAGTA TTTAGATGTTTCGCTTTGG	V30	60 ºC	30	[10]
V36F V36R	TGGTTCTTGGGGTAATTCTGTT CTACCAGGAGATTATCAAAACGAA	V36	58ºC	30	[9]
V36NF V36NR	TGGCGTCGAAGACAAA ACTCTACCAGGAGATTATCAAA	V36	60 ºC	25	[10]
V50F V50R	CTTGTTGGATCACAATACGAACTATA GGTAAGGGACAAAGTAAGTGAAGC	V50	56ºC	30	[9]

2.4. Data Analysis

Using the Quantity One 1D Analysis software package (BioRad), the agarose gel images were analyzed and the allelic sizes were estimated. The sizes were then converted into an allele designation, as shown in Table 4, which in turn formed the allele string for the five loci. The allele string was constructed in the following order: V27-V27N-V29-V29N-V30-V30N-V36-V36N-V50. Each unique allele string was given a unique MLVA type (MLVA) number (Table 5).

Table 4 Allele designation for tested *Leptospira* spp.

Allele Designation	Size (bp) of: amplicon from loci/primers								
	V27		V29		V36		V36		V50
	V27F/R	V27NF/R	V29F/R	V29NF/R	V30F/R	V30NF/R	V36F/R	V36NF/R	V50NF/R
1	201	216	149	386	264	388	227	195	283
2	252	265	202	424	307	425	240	226	347
3	311	339	231	454	340	452	270	260	387
4	371	369	325	481	372	492	305	291	417
5	422	409	375	535	443	521	365	360	442
6	469	465	411	592	480	558	392	406	475
7	494	491	435	639	541	588	418	484	519
8	531	540	490	663	584	639	452	525	540
9	579	596	614	706	673	685	489	572	564
10	612	637	918	771	714	746	546	605	623
11	643	704		866	790	790	621	634	
12	671			908	916	819	675	738	
13	722			1182	1175	878	713	790	
14					1359	918	788	1137	
15						950	815		
16						983	865		
17							1171		

Table 5 MLVA patterns of *Leptospira* reference serovar

MLVA Pattern	Serovars	V27	V27N	V29	V29N	V30	V30N	V36	V36N	V50N
1	Autumnalis	7	7	4	7	12	15	12	9	1
2	Australis	7	0	4	0	12	0	0	0	0
3	Ballum	6	0	4	0	12	15	13	9	0
4	Bataviae	12	11	8	10	3	3	3	2	8
5	Bratislava	8	0	0	0	12	13	4	0	0
6	Bulgariga	0	0	0	8	5	0	0	10	0
7	Canicola	1	1	3	5	9	10	16	13	3
8	Cynopteri	5	6	1	2	1	1	10	7	5
9	Djasiman	12	11	5	8	8	8	6	5	7
10	Fortbragg	2	2	5	8	2	3	5	4	5
11	Grippotyphosa	6	7	1	2	2	3	1	1	1
12	Hebdomadis	11	11	1	2	9	9	16	13	4
13	Icterohaemorrhagiae	0	0	0	0	0	0	0	0	0
14	Javanica	5	5	3	4	2	2	15	11	4
15	Mini	12	11	2	3	13	16	11	8	2
16	Nanla	3	3	7	8	6	7	4	3	0
	New	3	3	7	8	6	7	4	3	0
	Panama	3	3	7	8	6	7	4	3	0
17	Pyrogenes	12	10	1	1	9	10	12	10	6

Table 5 MLVA patterns of *Leptospira* reference serovar (cont')

MLVA Pattern	Serovars	V27	V27N	V29	V29N	V30	V30N	V36	V36N	V50N
19	Ranarum	8	6	4	6	11	14	12	10	1
20	Saigon	4	3	8	10	4	6	17	14	5
21	Sarmin	4	3	10	13	2	3	15	11	3
22	Sejroe	10	0	0	0	8	0	0	12	0
23	Shermani	4	4	6	9	9	10	9	5	0
	Wolffi	13	9	6	9	15	0	9	5	4
	Celledoni	0	0	0	0	14	0	0	0	0
	Mujunkummi	0	0	0	0	0	0	0	0	0
	Pomona	0	0	0	0	0	0	0	0	0
	Naam	0	0	0	0	0	0	0	0	0
	Patoc	0	0	0	0	7	0	0	11	0

3. Results

3.1. Detection of VNTR by PCR

Figure 1 shows representative VNTR loci allele of reference serovar and patient isolates. The representative amplicons of all nine loci (designated V27, V27N, V29, V29N, V30, V30N, V36, V36N, V50) detected in five genomospecies of *Leptospira* reference serovars *i.e. L. borgpeterseni*, *L. interrogans*, *L. Kirshneri*, *L. noguchii*, *L. Weilii* were randomly selected and confirmed by DNA sequence analysis.

Thirty *Leptospira* reference serovars revealed 23 distinct MLVA patterns designated MLVA-1 to 23 (Table 5). Three serovars (Nanla, New and Panama) had identical MLVA type, MLVA-I6. The MLVA pattern was not assign to five *Leptospira* serovars *i.e.* Celledoni, Mujunkunmi, Pomona, Naam and Patoc) as they yielded less than 3 VNTR loci amplicons from the nine primer pairs used. Tables 4-6 give details on the string number of MLVA alleles of VNTR loci of each MLVA patterns and also serovars of the particular MLVA patterns.

3.2. Serovar identification of *Leptospira* patient isolates by MLVA

The 55 *Leptospira* isolated from Thai patients during 2002 to 2006 showed an addition of other 15 MLVA patterns, designated MLVA-24 to 38 (Table 6).

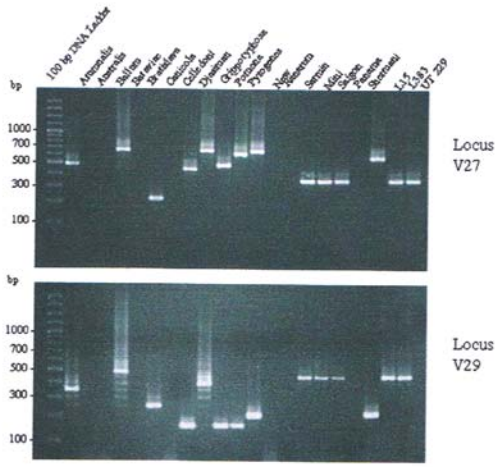

Figure 1 Amplicons obtained from the V27 and V29 loci of various Leptospira reference strains and clinical isolates (L15, L393 and U229)

Thirty-two patient isolates were identified as serovar Autumnalis by MAT and latex agglutination (Table 2). Among these, 25 isolates had MLVA-24 and only one isolate had MLVA-1 similar to that of the reference Authumnalis serovar. The remaining six isolates which identified as serovar Authumnalis by MAT gave MLVA-25 pattern. The other 18 patient isolates gave unique MLVA patterns compared to that of the reference serovars (Table 5 and 6).

Table 6 MLVA patterns of *Leptospira* spp. Thai patient isolates

MLVA pattern	Serovars		V27	V27N	V29	V29N	V30	V30N	V36	V36N	V50N
	MAT	Latex									
24	Autummnalis	Autumnalis	9	9	2	3	4	4	2	2	5
25	Autummnalis	Autumnalis	10	10	9 or 1	2 or 4 or 10	3	3 or 6	1	12	4
26	Bataviae	ND	3	3	8	10	3	3	15	12	5
27	Bataviae	ND	12	11	8	10	3	3	4	3	8
28	Erincciauriti	ND	0	0	0	5	0	0	0	2	0
29	Korat	ND	0	0	2	0	4	0	2	2	0
30	Mini	ND	9	5	6	9	5	7	4	3	0
31	Medanensis	ND	3	4	8	10 or 11	4	4	15	12	5
32	Pyrogenes	ND	7	8	2	2	2	3	2	2	1
33	Pyrogenes	ND	9	9	9	12	11	11	13	12	6
34	Pyrogenes	ND	9	10	9	12	4	5	15	13	4
35	Saigon	ND	9	5	6	9	5	7	4	3	0
36	Sejroe	ND	0	0	2	0	6	0	7	0	0
37	Sejroe	ND	9	9	9	12	10	12	3	2	10
38	Weerasinghe	ND	9	4	6	8	8	10	8	6	0

ND; not determined

4. Discussion

Leptospirosis is a re-emerging zoonosis of the tropical and subtropical areas of the world. It is also a major health problem in Thailand. *Leptospira*, the causative agent of Leptospirosis, can be classified into more than 300 serovars based on serological technique [1, 12]. To date, MAT is the major means to identify *Leptospira* serovars for epidemiological tracing. MAT is a sophisticated technique, relying on special equipment and expertise. Moreover, MAT involves a large panel of serovar-specifc antisera. Its preparation employs several hundred laboratory animals (e.g. rabbit), which is always prone to animal ethics debate. The laboratory personnel are also endangered by infection from the requirement of maintaining the development of a new and simpler technique is a priority. Although, molecular techniques such as restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) have been helpful, similar to MAT, these methods are time consuming and require expensive equipment and expertise similar to that of MAT [4]. VNTR has been described previously in several organisms including *Leptosira* spp. [6, 7, 9, 10]. The better discriminatory power and more accurate determination of genetic relatedness is achieved based on analyzing multiple VNTR loci simultaneously, so called MLVA [9, 10, 11]. The literature shows a distinct MLVA pattern for each *Leptospira* reference serovar. Therefore MLVA appears to be a convincing method for characterization and differentiation of *Leptospira* serovars. Recently, VNTR loci V4, V7, V9, V10, V11, V19, and V23 had been demonstrated to be able to distinguish 53 of *L. interogans* reference serovars with the exception of serovars that are predominately in the Southeast Asian region, *e.g.*, Australis,Bratislava, Copenhagani, and Icterohaemoragiae [9]. Moreover, V27, V29, V30, V36, and V50 loci also have been elucidated to possess an impressive discrimination power in typing of 10 reference serovars, which are present in Australia and Thailand [10]. Nevertheless,

their effectiveness in patient isolate serovar identification has been shown only for the serovar Australis. In this study, the usefulness of the mentioned above VNTR loci in *Leptospira* serovar typing were successfully expanded to the differentiation of an additional 15 reference serovars that are predominately in Thailand. The patient isolates belong to serovars other than Australis. Furthermore, it was found that patient isolates belonging to serovar Autumnalis and Pyogenes provided multiple varieties of MLVA patterns that are unique to them. As observed by other investigators, this technique is likely to be a straightforward approach to the clonal structure of bacterial populations [6]. This indicates that *Leptospira* serovar Autumnalis and pyogenes strains are not a single worldwide epidemic clone, but a simultaneous presence of several epidemics clones. It is likely that *Leptospira* serovar has undergone and continues to undergo evolution. Our study also confirms the high degree of reproducibility of the MLVA technique, as previously reported, MLVA patterns of the same reference serovars are also evidence for this. Although, many of MLVA patterns represented in the Thai isolates were not identical to that of their respective reference serovars used in this study, they were closely related to the MLVA patterns of the respective serovars found in previous studies [9, 10]. In addition, it was found that many of Autumnalis serovars isolated from Thai patients of the same regions revealed different and new MLVA patterns such as MLVA-24 and 25, indicating the diversity of the endemic strains in our country. Analysis of five VNTR loci revealed additional 15 different MLVA patterns among the 55 Thai clinical isolates, apart from Autumnalis and Pyogenes, serovars Bataviae, Geippotyphosa, Hebdomadis, Mini, Sejroe and Saigon and Shermani gave MLVA patterns that were distinctive to their respective reference serovars, suggesting the presence of diverse epidemic clone.

This study further emphasizes that MLVA is a powerful method for simple serovar typing. Furthermore, the appearance of the new MLVA patterns for the similar serovars in Thailand further highlight the fact that MLVA can be used for the defined identification at the clonal level. Therefore, MLVA can be used to construct a database that is not only useful for serovar identification, but also can be used for the epidemiological tracing of the epidemic strains. In addition, it also can be used as a mainstay for constant monitoring of the genetic characteristic changes of *Leptospira* in the same region and different part of the world to trace the epidemiological pattern of the existing pathogenic clone and recognizing newly emerged/imported variants.

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