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The isolated *Leptospira Spp.* Identification by molecular biological techniques

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ABSTRACT

Leptospirosis is a zoonotic disease caused by the bacteria of *Leptospira* spp. Identification of this bacterium relies on serotyping and genotyping. Data base for animal causative serovars in Thailand is limited. As the unknown serovars are found in the laboratory, they need to be sent overseas for referent identification. To reduce the cost, this research intended to develop a leptospiral identification method which is user–friendly and able to classify efficiently. Ten *Leptospira* isolations were cultured from urine samples. They were identified by three molecular biological techniques, including Pulsed-Field Gel Electrophoresis (PFGE), Variable Number Tandem Repeat (VNTR) and Multilocus Sequence Typing (MLST). These methods were developed and compared to find the most suitable one for leptospiral identification. VNTR was found to be inappropriate since it could not identify the agents and it did not show the PCR product. PFGE and MLST gave the same results of the unknown 1 and 2 which were *L.weilii* sv Samin st Samin. Unknown 4 showed different results by each technique. Unknown 5 to 10 were likely to be L.meyeri sv Ranarum st ICF and *Leptonema illini* sv Illini st 3055 by PFGE but MLST could not identify the serovar. However, molecular biological technique for *Leptospira* identification should be done by several methods in order to confirm the result of each other.

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INTRODUCTION

Leptospirosis is a zoonotic disease found globally especially in the tropical countries like Thailand.[1] This becomes a public health problem with animal reservoir especially the rodents.[2,3] Wetland and aquatic migratory birds also carry and transmit leptospires.[4] The bacteria can also be found in other animals either in sylvatic or farm animals such as cattle, buffaloes, goats, sheep, pigs, dogs, rodents, reptiles, birds, and etc.[5, 6] Etiology of the disease is the infection by the leptospiral bacteria.[7] Identification of the leptospires is by either serotyping or genotyping methods. Serotyping can differentiate *L. interrogans*, the pathogenic, from *L. biflexa*, the non-pathogenic which live in natural water and soil. Genotyping can differentiate the leptospires into three groups, namely pathogenic, intermediate and non-pathogenic agents. These two techniques have no correlation to each other.[5]

Data of leptospirosis infection in livestock in Thailand seem to be much underestimated. As most animals are asymptomatic or have mild symptoms. Furthermore, only the clinical symptoms of the disease could not be differentiated from others.[8] WHO recommends microscopic agglutination test (MAT) which detect the antibody in serum to be used

in livestock laboratory diagnosis as well as polymerase chain reaction (PCR) by culturing and examining the leptospiral genome.[9] However, antibody detection by MAT could not diagnose recent infection since detectable titers of antibody generally become detectable only 5 to 7 days after onset of illness.[10,11] In addition, culturing which take time for 2-3 months might give the false negative results.[5, 12] Examining the leptospiral genome by PCR can indicate only the leptospires found are pathogenic or not. This is why animal serovar database of pathogenic strains in any areas is so scarce and could not relate to human disease. This problem becomes obstacle for prevention and control planning of the disease along with the appropriate specific test kit and vaccine development in each region.

Owing to that, our group intended to develop a convenient and practical leptospiral diagnostic method by molecular biological techniques, including Pulsed-Field Gel Electrophoresis (PFGE), Variable-Number Tandem-Repeat (VNTR) and Multilocus Sequence Typing (MLST). These popular techniques would be developed and compared that aimed for appropriate leptospiral identification method.

MATERIALS AND METHODS

Leptospire Isolation

Leptospires from urine samples sent to Leptospirosis Center were cultured by diluting the urine samples with 1% bovine serum albumin (BSA) and put into the culture media of Johnson and Harris modification of the Ellinghausen and McCullough medium (EMJH) (Difco, USA). There are two types of EMJH media, namely semi-solid and solid medium. The bacterial culture were incubated at 30°C. The presence of the leptospires was checked weekly for 12-16 weeks by dark field microscope. Suspected samples would be subcultured by putting into the new EMJH broth and the bacteria were purified by using 0.2 µm–pore–size membrane filter. Then they were observed under the dark field microscope. The presence of *Leptospira* spp. was considered as positive examined by dark field microscopy for observing the characteristic of thin helical structures with prominent hooked ends and motility.[13]

Preparation of *Leptospira* Samples for Screening and Identification

DNA of the cultured leptospires were extracted by preparing ten cultured samples at the concentration of 1×10^6 cells/ml, and then washed with 1XPBS. The cell precipitations obtained were extracted by QIAamp DNA Mini Kit (Qiagen, Netherlands). DNA samples were kept at -20°C until use.

Leptospiral Screening by Real Time PCR

Twenty microliter of the master mix which was prepared from Fast Start essential DNA green master (Roche, Switzerland). The primer sets were designed for detection of 16S rRNA gene of all *Leptospira* species (nonpathogenic, intermediate, pathogenic).[14] Then it was amplified by LightCycler® nano instrument (Roche, Switzerland) at 45 cycles.

Pulsed Field Gel Electrophoresis (PFGE)

The referent and cultured leptospires were prepared at the concentration of 10^8 cells/ml in EMJH broth. Leptospiral DNA was prepared according to the instruction manual of CHEF Bacterial Genomic DNA Plug kit (Bio-Rad Laboratories, United Kingdom). DNA were cut by Not I restriction enzyme (New England Biolabs, USA) at 37°C overnight.

DNA were separated on gel with 120 Volt AC at the angles of +60 and -60 degree by CHEF MAPPER (Bio-Rad Laboratories, United kingdom) in 0.5X TBE at 14°C for 24-28 hours. The initial and final switch time were 5.5 and 63.5 seconds, respectively.

Patterns of the leptospiral DNA were analyzed and compared with those of the referent leptospires by the Gel compare II program (version 4.6; Applied Maths, USA). The cluster analysis of Dice band-based coefficient dendogram was constructed by Unweighted Pair Group Method with Arithmetic (UPGMA).

Variable-Number Tandem-Repeat (VNTR)

Five pairs of primers, including VNTR4, 7, 10, Lb4 and Lb5 were used.[15] The 50 µl of master mix composed of Nuclease-free water, 2X Go Taq green master mix (Promaga, USA), forward primer and reverse primer as well as 5 µl of DNA primer were performed. Amplification of the genetic materials was done by

Thermocycler (HVD life science, Austria) at 30 cycles. The PCR products and copy numbers obtained was then compared with the references.

Multilocus Sequence Typing (MLST)

Seven housekeeping gene, namely *tipA*, *sucA*, *caiB*, *pntA*, *pfkB*, *glmU* and *mreA* were used.[16] The master mix of 50 µl composed of Nuclease-free water, 2X Go Taq green master mix (Promaga, USA), 1.5 mM-2.5mM $MgSO_4$, forward primer and reverse primer together with 5 µl of DNA primer were performed. Amplification was done by Thermocycler (HVD life science, Austria) at 30 cycles. PCR product sequencing and base sequenced analysis was done by MEGA6 program. Then identification of the pathogenic leptospires was performed by comparing with the database in http://leptospira.mlst.net/.

RESULTS

PFGE

DNA fingerprints of the unknown 1 and 2 were similar to the referent leptospira of *L. weilli* serovar (sv) Sarmin Strain (st) Sarmin at 100 % similarity. Whereas the unknown 3 had the DNA fingerprint like *L. weilli* sv Celledoni st Celledoni and *L. borgpetersenii* sv Javanica st Veldrat Bataviae 46 reference at similarity of 85.7 % and 80.6 %, respectively. That of the unknown 4 was closed to *L. interrogans* sv Bataviae st Swart reference at similarity of 72 %. Meanwhile, the unknown 8 and 9 had the fingerprints that were 100 % similarity. The unknown 6, 7 and 10 were 100 % similarity and also were similar to those of the unknown 8 and 9 at 88.9 % similarity. These 5 unknowns were classified as a group of which fingerprints were similar to the reference of *L. meyeri* sv Ranarum st ICF and *Leptonema illini* sv Illini st 3055 with 81.2 % similarity. Besides, the unknown 5 also had the fingerprint similar to those of the 5 unknowns and *L. meyeri* sv Ranarum st ICF as well as *Leptonema illini* sv Illini st 3055 with 64.7 % similarity. Figure 1 shows Patterns of DNA fingerprint of the leptospiral unknown 1-10 compared to the leptospiral references cut by Not I restriction enzyme. Size classification by Pulsed Field Gel Electrophoresis technique and Gel compare II program with 2.0 % band position tolerance as well as 1.5 % optimization value. Similarity of the DNA fingerprint was calculated by Dice coefficient and dendrogram was constructed by UPGMA.

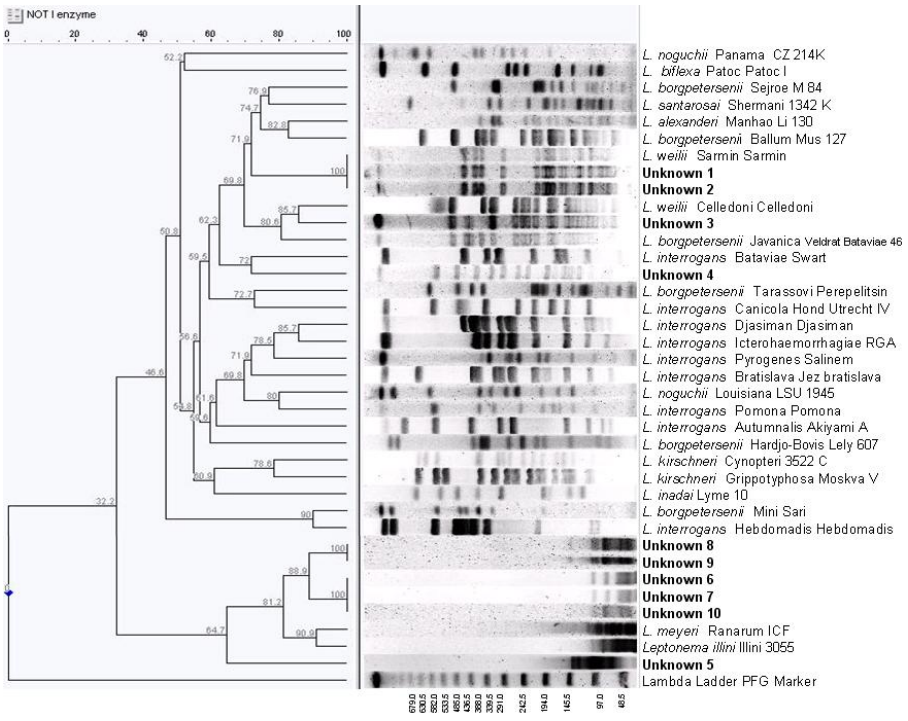

Fig 1 Patterns of DNA Fingerprints of the Leptospiral Unknown 1-10.

VNTR

VNTR could not differentiate pathogenic leptospires of the unknown 1 to 10 since there were 5 unknowns that did not show the PCR product (unknown 6, 7, 8, 9 and 10). Besides, the unknown 1, 2, 3, 4 and 5 did not have a clear form of the copy number or did not conform to the references in the database. In addition, the unknown 1 and 2 gave the same results which provided the PCR products of Lb4 and Lb5 with the copy number of 7 and 5, respectively while the unknown 3 also gave the same PCR products as those of the unknown 1 and 2 with the copy number of 4 and 2, respectively. Concerning the unknown 4, it showed the PCR products of VNTR10, Lb4 and Lb5 with the copy number of 4, 4 and 2, respectively. After all, the unknown 5 provided the PCR product of Lb5 with the copy number of 5. (Fig 2 and Table 1)

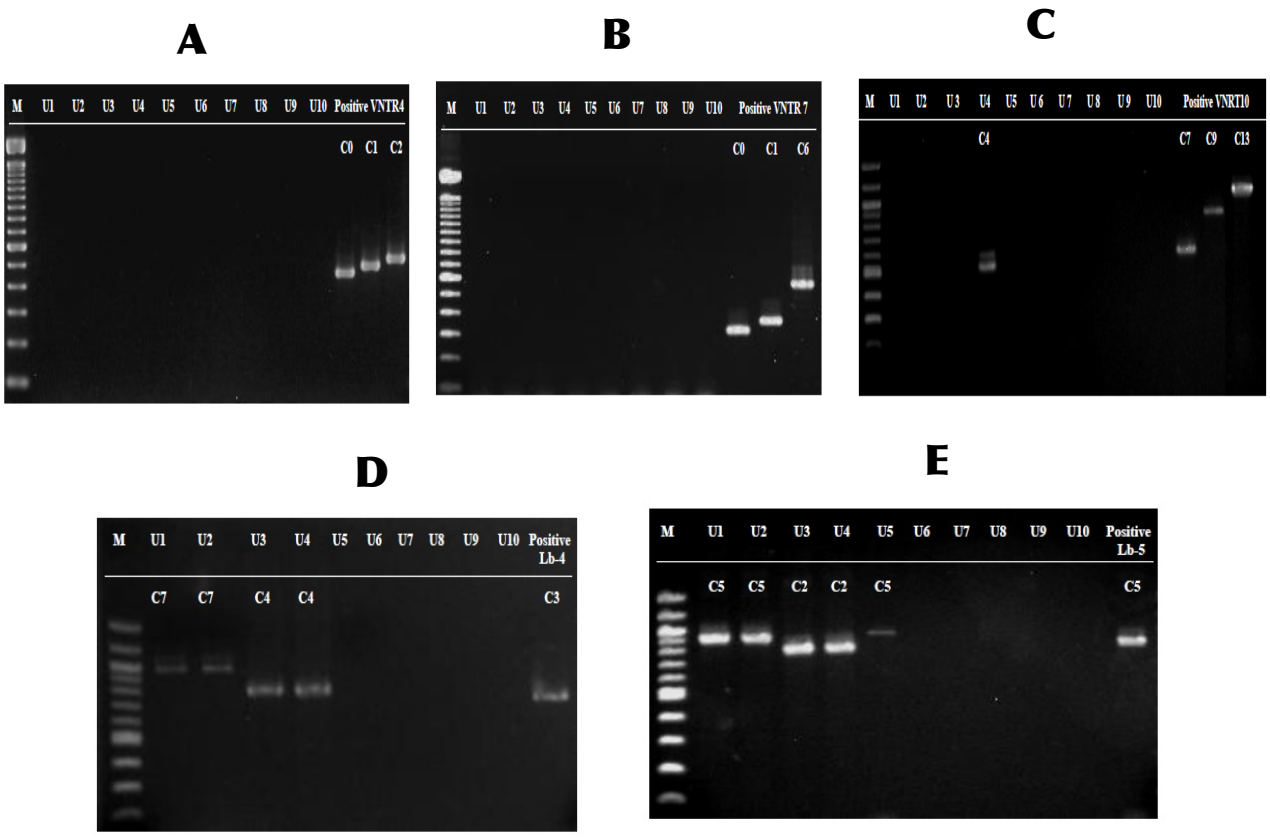

Fig 2 PCR product of VNTR4, VNTR7, VNTR10, Lb4 and Lb5, respectively.

(C, copy number; M, DNA ladder; U, unknown samples)

Positive controls of each primer derived from different species of Leptospira as follows: picture A: Co, L. kirschneri sv Cynopteri st 3522 C: C1, L. interrogans sv Canicola st Hond Utrecht IV; C2, L. interrogans sv Pomona st Pomona. Picture B: Co, L. interrogans sv Pomona st Pomona; C1, L. interrogans sv Icterohaemorrhagiae st RGA; C6, L. kirschneri sv Cynopteri st 3522 C. Picture C: C7, L. interrogans sv Icterohaemorrhagiae st RGA, C9, L. interrogans sv Autumnalis st Akiyami A, C13, L. interrogans sv Pyogenes st Salinem. Picture D: C3, L.borgpetersenii sv Sejroe st 1342 K. Picture E:C5, L. borgpetersenii sv Sejroe st 1342 K.

Table 1 Displaying the Copy Numbers of the PCR Products of Primer VNTR 4, 7, 10, Lb4, Lb5 by VNTR which cannot differentiate the Unknown 1 to 10

Unknown Sample	Copy Number				
	4	7	10	Lb4	Lb5
1	ND	ND	ND	7	5
2	ND	ND	ND	7	5
3	ND	ND	ND	4	2
4	ND	ND	4	4	2
5	ND	ND	ND	ND	5
6	ND	ND	ND	ND	ND
7	ND	ND	ND	ND	ND
8	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND

ND, no PCR product

MLST

The unknown 1, 2, 3 and 4 gave PCR product of all 7 genes. The unknown 5 showed PCR products of 2 genes, namely *TpiA* and *mreA* whereas the unknown 6, 7, 8, 9, and 10 gave no PCR product of any gene. The unknown 1 and 2 performed the same results of all 7 genes, including *tipA, glmU, caiB, pntA, sucA, mreA* and *pfkB* with allele numbers of 54, 50, 48, 62, 58, 53 and 65, respectively. It was found that the Sequence Type (ST) 191 was matched with those of *L. weilii* sv Samin st Samin. Meanwhile, the genes of the unknown 3, including *tipA, glmU, caiB, pntA, sucA, mreA* and *pfkB* had allele numbers as 34, 24, 11, 27, 30, 27 and 67, respectively which were matched with ST143, namely *L. borgpetersenii* sv Javanica st Veldrat Batavia 46. In the unknown 4, the sequencing result of genes, including *tipA, glmU, caiB, pntA, sucA, mreA* and *pfkB* demonstrated the allele numbers as 35, 26, 29, 30, 28, 29 and 39, respectively which were matched with ST 152, namely *L. borgpetersenii* sv Hardjo-bovis st JB 197. The unknown 5 displayed PCR products of 2 genes, *tpiA* and *mreA*. After analysis of nucleotide sequences, 2 allele numbers of 54 and 53 were found, respectively. Since there was the information of only 2 genes, it was then unable to identify the ST, species, serogroup, serovar and strain. (Table 2)

Table 2 Result of allele number of PCR product of 7 genes, namely tipA, glmU, caiB, pntA, sucA, mreA and pfkB by MLST

Unknown Sample	allele number							ST	Identity		
	tpiA	*glmU*	*caiB*	*pntA*	*sucA*	*mreA*	*pfkB*		species	serovars	strains
1	54	50	48	62	58	53	65	191	*L. weilii*	Sarmin	Samin
2	54	50	48	62	58	53	65	191	*L. weilii*	Sarmin	Samin
3	34	24	11	27	30	27	67	143	*L. borgpetersenii*	Javanica	Veldrat Batavia 46
4	35	26	29	30	28	29	39	152	*L. borgpetersenii*	Hardjo-bovis	JB197
5	54	ND	ND	ND	ND	53	ND	-	-	-	
6	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
7	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
8	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
9	ND	ND	ND	ND	ND	ND	ND	-	-	-	-
10	ND	ND	ND	ND	ND	ND	ND	-	-	-	-

ND, no PCR product

After comparing all 3 molecular biological identification methods of the unknown 1 to 10, it was found that VNTR could not identify leptospire serovar whereas PFGE and MLST provided the same results of the 2 samples which were the unknown 1 and 2, namely *L. weilli* sv Samin st Samin. For the unknown 5 to 10, PFGE was able to identify them as sv Ranarum/Illini. MLST could not identify the leptospire serovar. (Table 3)

Table 3 Comparison of Molecular Biological Identification of the Unknown 1 to 10 by PEGE, VNTR and MLST

Unknown Sample	PFGE		VNTR	MLST		
	Serovars	%Similarity		Species	Serovar	Strain
1	Sarmin	100.0	ND	*L. weilii*	Sarmin	Sarmin
2	Sarmin	100.0	ND	*L. weilii*	Sarmin	Sarmin
3	Celledoni	85.7	ND	*L. borgpetersenii*	Javanica	Veldrat Batavia 46
	Javanica	80.6				
4	Bataviae	72.0	ND	*L. borgpetersenii*	Hardjo-bovis	JB197
5	Ranarum/Illini	64.7	ND	-	-	-
6	Ranarum/Illini	81.2	ND	-	-	-
7	Ranarum/Illini	81.2	ND	-	-	-
8	Ranarum/Illini	81.2	ND	-	-	-
9	Ranarum/Illini	81.2	ND	-	-	-
10	Ranarum/Illini	81.2	ND	-	-	-

DISCUSSION

From all 3 molecular biological identification methods of the 10 samples, it was found that PFGE could identify all samples observed, MLST could identify 4 samples (the unknown 1 to 4) and VNTR could not, since there was no PCR product. There were only 5 samples that revealed the PCR product but the copy number patterns were either not clear or did not match with the references in the database. For the unknown 1 and 2, VNTR could only indicate the possibility that both were pathogenic strains that were the same or closely related since they had the same pattern of the copy number. MLST which was pathogenic strain identification could select the target DNA with regular expression (housekeeping gene) that scattered all over genome of the leptospire. This type of gene will not change the nucleotide sequence (mutation) or with minor change. At present, there are 7 target DNA which are *tipA*, *sueA*, *caiB*, *pntA*, *pfkB*, *glmU* and *mreA* gene. These are specific in 7 pathogenic leptospire species, including *L. interrogans*, *L. borgpetersenii*, *L. kirschneri*, *L. weilii*, *L. santarosai*, *L. noguchii* and *L. alexanderi.*[16] MLST method suits for *Leptospira* identification which shows completely PCR products of seven target genes therefore, the results of the unknown 5 to 10 were not able to be classified. The unknown 3 was classified as *L. borgpetersenii* sv Javanica st Veldrat Batavia 46. Considering this referent DNA fingerprint pattern by PFGE, it was found that sv Javnica (similarity 80.6 %) had more similar pattern than that of sv Celledoni which had 85.7 % similarity. MLST result of the unknown 4 was *L. borgpetersenii* sv Hardjo-bovis st JB 197 but PEGE gave the similar result with sv Bataviae which had 72.0 % similarity. This was quite low but the similarity with sv Hardjo-bovis was still less than sv Bataviae. Therefore, these two techniques provided obviously different results.

Identification of the leptospire by molecular biological techniques such as VNTR, PFGE and MLST manifested limitations of each technique. VNTR could be used with few leptospire reference[15] and could give good results if the unknown was *L. interrogans* and *L. kirschneri.*[15, 17] This technique did not give the clear result because same pattern of the copy number made it impossible to identify the serovar. Some serovars share 99% nucleotide

similarity and furthermore the polymorphisms were found by VNTR study led to the absence of the PCR product in some pathogenic species or serovar.[18,19] Result analysis was difficult for VNTR since PCR product size on the agarose gel needed to be compared roughly with DNA ladder and might easily lead to error. In this study, five primers for VNTR loci were specific for three species namely, *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii.*[15] However, whenever VNTR was used to identify the pathogenic leptospires, basic information such as species data should have been obtained. If there was no information related to species, other techniques should be applied.

PFGE technique uses enzyme to cut the leptospire DNA and compare the DNA fingerprint patterns with the reference. This could be used to study molecular biological epidemiology by observation of the variation. If the patterns are not in the referent list then this is not practical.[20] However, MLST might not relate to serovar such as Pomona and Canicola serovars which both have the same ST 37 or Pyrogenes and Grippotyphosa serovars which have many ST even though they are the same serovar. PFGE could solve this problem of identification.[21] In conclusion, PFGE relies on observation of chromosome arrangement while MLST was used for strain phylogeny.[22] Nowadays the pathogenic leptospires in MLST database possess 209 ST. There should be some pathogenic leptospires that MLST could not cover such as *L. alstonii* and *L. kmetyi*. However, molecular biological technique for *Leptospira* identification should be done by several methods in order to confirm the result of each other.

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