การตรวจหาวัวที่เป็นแหล่งรังโรคของเชื้อเลปโตสไปโรซีสในประเทศไทยโดยการประสานเทคนิค PCR และ MAT

PCR-Based and MAT Combination Methods for Identifying Leptospira Reservoir Cows in Thailand

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บทคัดย่อ

คณะผู้วิจัยได้ใช้เทคนิค microscopic agglutination test (MAT) เพื่อตรวจระดับ titer ต่อโรคเลปโตสไปโรซีส และใช้เทคนิค Polymerase Chain Reaction (PCR) เพื่อตรวจสอบ DNA ของเชื้อเลปโตสไปร่า จากปัสสาวะโค ในพื้นที่ กรุงเทพมหานคร ปทุมธานี นครปฐม และ นครราชสีมา เทคนิค PCR ที่ใช้ในการศึกษาครั้งนี้ใช้ชุด primers ที่ได้รายงานโดย Gravekamp และคณะ (1993) ได้ทำการตรวจสอบเชื้อเปรียบเทียบของเชื้อเลปโตสไปร่า จำนวน 24 strains (24 serovars) และเชื้อเลปโตนีมา 1 ชนิด ผลการตรวจสอบเชื้อเปรียบเทียบโดยวิธี PCR คณะผู้วิจัยพบว่าเชื้อเปรียบเทียบบางตัวให้ผล PCR แตกต่างจากที่รายงานโดย Gravekamp และคณะ (1993) ผลการตรวจสอบ MAT จากซีรัม พบระดับ titer $\geq$1:40 มีอัตราสูงมากที่ 41% โดยการประสานเทคนิค PCR เพื่อตรวจสอบ DNA ของเชื้อเลปโตสไปร่า จากปัสสาวะโค ร่วมกับวิธี MAT พบว่ามีโคจำนวน 17.8% ที่จัดอยู่ในกลุ่มที่เป็นแหล่งรังโรคของเชื้อเลปโตสไปร่า ด้วยการผสมผสานสองเทคนิค ทำให้ได้ผลการตรวจที่เชื่อถือได้เพื่อการกำจัด หรือ เพื่อจำกัดพื้นที่ของโคในกลุ่มนี้

ABSTRACT

We did microscopic agglutination test (MAT) and PCR-based method to detect *Leptospira* titer in serum and *Leptospires* DNA in urine samples. Serum and urine samples were collected from the same cows at area Bangkok, Pratumdranee, Nakornprathom, and Nakornrachaseema. The PCR was performed with primer sets reported by Gravekamp et al. (1993) to check *Leptospira* DNA from 24 strains (24 serovars) and one *Leptonema* spp. There was some discordance of our PCR results to that reported by Gravekamp et al (1993). The percentage of cows is large (41%) with seropositive titer at $\geq$ 1:40. However, by using the combination methods of MAT and PCR, there were only 17.8% cows detected as *Leptospira* carrier and shedder. With these combination methods, we are confident in making decision to eliminate and/or to quarantine those animals.

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INTRODUCTION

Leptospirosis is a common disease in wildlife and livestock. Infection in livestock causes serious economic loss. It is a contagious disease causing abortion in 25-30% infected cows (Radostits et al., 1994). Leptospiral mastitis was also reported (Ellis et al., 1976; Higgins et al., 1980). In Thailand, incidences of leptospiral abortion in cattle herd and dairy farm were sporadically reported in 1997 and 1999 (Kingnate, 2000; Ratanamangkalanontra et al., 1999). The dairy cattle and feedlot cattle are also recognized as a reservoir for this microorganism (Radostits et al., 1994; Radostits et al., 1997; Yaeger and Holler, 1997). The percentage of leptospire infection among Thai population is increase 42 times from 1995 to 1999 (Data from Department of Epidemiology, Ministry of Public Health, 1999). Despite this increasing rate, there is no difference in the percentage of leptospiral seropositive cows and buffaloes reported in the leptospire epidemic areas and the leptospire non-epidemic areas (Suwanchareon et al., 2000). The microscopic agglutination technique (MAT) is a standard serological test utilized routinely at the National Institute of Animal Health (NIAH). The drawbacks of MAT are that it is not sensitive enough to test the infected cows at the early phase of infection. In addition, the high titer can be detected in cows receiving antigen long time ago (Radostits et al., 1994; Radostits et al., 1997). In general, the occurrences of seronegative carriers (Hathaway et al., 1986) and seropositive non-excreting animals (Gerritsen et al., 1993; Gerritsen et al., 1994) make a limited useful of MAT. The MAT also is unable to distinguish titers due to vaccination and titers due to a chronic infection. The PCR-based assays have been established for detecting leptospire antigens from blood, CSF, urine and other clinical samples (Faber et al., 2000; Gerritsen et al., 1991; Gravekamp et al., 1993; Letocart et al., 1997; Merien et al., 1992; Romero et al., 1998; Van Eys et al., 1989; Woo et al., 1997; Zuerner et al., 1995). This technique is sensitive, rapid. Up to now, neither simple PCR-based method is able to distinguish all leptospires up to the level of serovars. Additional more complicated PCR-based methods must be performed in order to identify pathogenic leptospire genospecies (Letocart et al., 1997; Woo et al., 1997; Zuerner et al., 1995). Clearly, the complicated methods are not suit for utilizing by laboratory in Thailand since expensive equipment, such as real-time PCR machine, are required. Our gold is to demonstrate method and combinatorial methods with fast, sensitive, and reliable results and not too expensive for identifying leptospiral carrier status in cows.

In this experiment, we designed a simple urine collection protocol for both PCR-based analysis and direct examination under dark field microscope. We made a combination of MAT, direct exam under dark field microscope, and PCR-based assay for evaluating cows' carrier status. This combination technique is sensitive, feasibility, and fast enough to evaluate cows' carrier status. The PCR-based technique performed in this experiment also can distinguish the pathogenic leptospires into 2 groups based on genospecies classification (Gravekamp et al., 1993).

MATERIALS AND METHODS

Bovine serum and urine samples were collected at area Bangkok, Pratumdranee, Nakornprathom, and Nakornrachaseema by Veterinarian officers and animal health officers. Serum samples were kept at -20 C until used for MAT. The MAT was performed with standard protocol (Faine et al., 1999; Suwanchareon et al., 2000). The routine 24 *Leptospira* serovars were tested in MAT by using 24 *Leptospira* reference live-antigens as shown in Table 2 and 3. Urine samples were prepared for the direct examination under darkfield microscope and PCR assay as followed. One ml of urine sample, collected as aseptic techniques as possible, was transferred to 9 ml of transport media (10 mg%5'FU, 1%BSA in PBS). The direct examination under dark field microscope was performed each day from day 1 till day 10. Under the dark field microscope, technicians can observe spirochetes, including leptospires, if the spirochete concentration is around 10^5 to 10^6 spirochete per ml. Once the samples were found positive, they were saved at 4 C for further sample preparation for PCR assay. At day 10 all negative samples were saved at 4 C for PCR sample preparation as well. One ml of urine sample in the transport media was used for PCR sample preparation. It was centrifuged at 13,000xg, 4 C, for 15 min. The supernatant was discarded and the pellet was washed with new fresh transport media. The washed pellet was saved by re-centrifuged at the same speed and period. The saved pellet was reconstituted with distilled water and boiled at 100 C for 15 min. The boiled sample was kept at -20 C to -40 C until PCR analysis performed. A 2 ul of boiled sample was used in the PCR reaction as described previously by Gravekamp et al. (1993) with a minor modification. Two sets of primers were used in PCR assay as detailed by Gravekamp et al. (1993). Briefly, the PCR reaction conditions were as follows in a total volume of 50 ul: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM each of dNTP, 50 pmol oligonucleotide primers, 1.25 U Taq DNA polymerase (Sigma). The PCR reactions were conducted in a thermocycler UNO-II (Biomatra) with the following cycle conditions: 94 C (30 sec), 40 sec at 55 C annealing temperature, and 72 C (45 sec) for 35 cycles. The *Leptospira* reference strains tested by PCR in this study were shown in TABLE 1.

RESULTS

We tested PCR primer sets as reported by Gravekamp et al. (1993). The *Leptospira*l strains tested in this study are shown in Table 1. Leptospires in almost genospecies gave a PCR product of 285 bp except the species of L. kirschneri gave a 563 bp PCR product as shown in Table 1 and Fig. 1. The L biflexa serovars andamana (strain CH11), maintenon (strain Maintenon), and patoc (strain Patoc 1), the L. meyeri serovar ranarum (strain ICF), and the Leptonema illini provided no PCR

product in the amplification reaction (Fig. 1 and Table 1). The L. biflexa serovar saopaolo (strain Sao Paolo) PCR product size is at around 240 bp. As shown in Table 1, the L. inadai serovar lyme (strain 10) has PCR product showed off at a correct size but the L. inadai serovar inadai (strain 92152-1) gave a bigger size of PCR product at around 340 bp.

TABLE 1. *Leptospira* reference strains used in this study.

Genospecies[a]	Serovar	PCR product size (bp)	Strain
L. interrogans	autumnalis	285	Akiyami A
	canicola	285	Hond Utrecht IV
	djasiman	285	Djasiman
	hebdomadis	285	Hebdomadis
	icterohaemorrhagiae	285	RGA
	pomona	285	Pomona
	pyrogenase	285	Salinem
L. noguchii	louisiana	285	LSU 1945
L. borgpetersenii	ballum	285	MUS 127
	javanica	285	Veldrat Batavia 46
	tarassovi	None	Perepelitsin
	sejroe	285	M 84
L. santarosai	shermani	285	1342 K
L. weilii	sarmin	285	Sarmin
L. kirschneri	cynopteri	563	3522 C
	grippotyphosa	563	Moskva V
L. biflexa	andamana	None	CH 11
	maintenon	None	Maintenon
	saopaolo	~240	Sao Paolo
	patoc	None	Patoc 1
L. meyeri	ranarum	None	ICF
	semaranga	285	Veldrat Semaranga
L. inadai	lyme	285	#10
	inadai	~340	92152-1
Leptonema sp.	illini	none	-

Note: [a] According to genetic classification of the genus *Leptospira* (Yasuda et al., 1987; Ramadass et al., 1992) in which the old pathogenic species L. interrogans and the saprophitic L. biflexa each were subdivided into a number of four non-pathogenic species and seven pathogenic species.

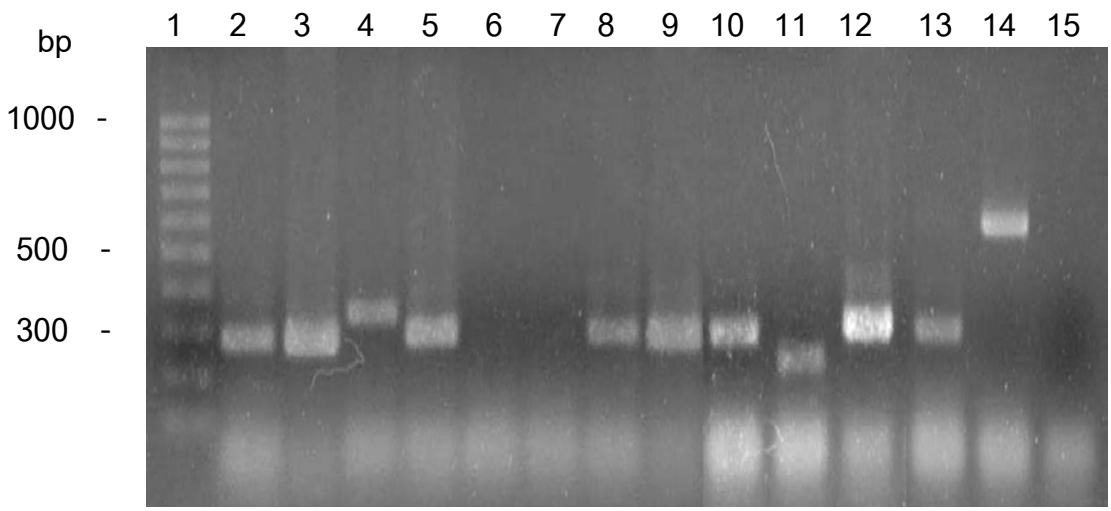

Fig. 1 Amplification of DNA showing PCR products of various serovars from pathogenic and non-pathogenic *Leptospira* species. DNA molecular mass markers (lane 1); autumnalis (lane 2); ballum (lane 3); inadai (lane 4); canicola (lane 5); leptonema (lane 6); patoc (lane 7); djasiman (lane 8); sarmin (lane 9); lyme (lane 10); saopaolo (lane 11); shermani (lane 12); louisiana (lane 13); cynopteri (lane 14); distilled water (lane 15).

TABLE 2. Total bovine serum and urine samples performed in this study by various techniques

	MAT [a]	Direct exam under dark field microscope	PCR technique
Positive	44	94	19
Negative	63	13	88
Total	107	107	107

Note: [a] Test on routine 24 *Leptospira* reference antigens to the following specific serovars at NIAH; bratislava, autumnalis, ballum, bataviae, canicola, celldoni, cynopteri, djasiman, grippotyphosa, hebdomadis, icterohaemorrhagiae, javanica, louisiana, manhao, mini, panama, pomona, pyrogenase, ranarum, sarmin, sejroe, shermani, tarassovi, patoc.

Serum and urine samples from 107 cows were tested by routine MAT, direct examination, and PCR-based method. As shown in Table 2, the direct examination under dark field microscope gave the highest positive result of 88% while the PCR technique gave 17.8% positive result counted as the lowest per-cent of positive result on samples from the same cows. The result from direct examination was not correlated with both MAT and PCR methods (Table 2). It was 80% of these direct examination positive cows that shown negative PCR result. It was also 54% of these direct examination positive cows that shown negative MAT titer. There are 41% and 59% of cows that shown MAT titer $\geq$ 1:40 and MAT titer of 0-1:20 to any serovars (Table 3). However, PCR technique detected only 17.8% of urine samples containing pathogenic *Leptospires* (Table 3). Comparing results

between MAT and PCR technique, It was only 30% of cows with MAT positive (41%) that shed leptospires into urine (Table 3). Nevertheless, genospecies grouping the leptospires, detected from cows' urine, based on PCR result were not matched with the sero-specific titer tested by MAT (data not shown).

TABLE 3. Relation and comparing between MAT and PCR techniques for *Leptospiral* diagnosis from total 107 bovine samples.

MAT[a] titer	PCR result (Percentage)		Total
	Pathogenic & ambiguity[b]	Non-pathogenic	
≥ 1:40	12.2	28.8	41
0 - 1:20	5.6	43.4	59
Total	17.8	72.2	100

Note: [a] Test on routine 24 *Leptospira* reference antigens to the following specific serovars at NIAH; bratislava, autumnalis, ballum, bataviae, canicola, celldoni, cynopteri, djasiman, grippotyphosa, hebdomadis, icterohaemorrhagiae, javanica, louisiana, manhao, mini, panama, pomona, pyrogenase, ranarum, sarmin, sejroe, shermani, tarassovi, patoc.

[b] The ambiguous PCR product size is around 630 bp. This is the size that is not comparable to PCR products derived from any *Leptospiral* reference strains shown in Table 1.

DISCUSSION

Our results on PCR technique to detect groups of genospecies, based on Gravekamp et al. (1993) primer sets, have some discordance with results reported by Gravekamp et al. (1993). Generally, PCR products amplified from DNA of L. interogans, L. noguchii, L. borgpetersenii, L. santarosai, L. weilii, L. kirschneri have the same size as reported by Gravekamp et al. (1993). However, we did not get PCR product from the amplification DNA of L. borgpetersenii serovar tarassovi (strain Perepelitsin) (Table 1). We triplicate performed the PCR reaction of this strain as similar technique as we did for other strains. We got positive result from a test of an agglutination reaction of this *Leptospira* strain with hyperimmune serum to serovar tarassovi (data not shown). The unexpected negative PCR result on this *Leptospira* stain must be further evaluation and be discussed with WHO/FAO/OIE Collaborating Center for References and Research for Leptospirosis, Western Region, Australia.

We found that L. biflexa serovar saopaolo (strain Sao Paolo) gave an unexpected PCR product of around 240 bp. However, we were confident that this unexpected PCR product was created by primers mismatch since we had already tested each primers sets. Neither of each primer sets alone could create the 240 bp PCR product from antigen of this strain (data not shown).

The L. inadai serovar inadai, strain 92152-1, gave a PCR product size of 340 bp while L. inadai serovar lyme, strain 10, gave the PCR product in a correct size as reported by Gravekamp et al. (1993). This is the first time in reporting the PCR result from L. inadai serovar inadai, strain 92152-1. Previously, Yasuda et al. (1987) and Ramadass et al. (1992) reported and classified this strain 10 as a pathogenic *Leptospira* species but Gravekamp et al. (1993) suggested a further investigation on this strain. Since, up to date, there is no document to confirm that L. inadai is not a pathogenic *Leptospira*, we'd count this *Leptospira* species as pathogenic Leptospira based on the classification by Yasuda et al. (1987) and Ramadass et al. (1992). Based on DNA homology, the leptospiral strains were divided into four non-pathogenic species, L. biflexa, L. meyeri, L. parva, and L. wolbachii, and seven pathogenic species, L, interrogans, L. borgpetersenii, L. Weilii, L. noguchii, L. santarosai, L. inadai, and L. kirschneri.

The PCR results of L. meyeri in this report were not concurred with those reported by Hookey (1992) and Gravekamp et al. (1993). Hookey (1992) and Gravekamp et al. (1993) found PCR products of the correct size from DNA of both *Leptospira* serovar ranarum (strain ICF) and serovar semaranga (strain Veldrat Semaranga), but we could not get any amplified PCR products from *Leptospira* serovar ranarum (strain ICF). This controversy makes a difficult interpretation since *Leptospira* serovar ranarum (strain ICF) and serovar semaranga (strain Veldrat Semaranga) were designated as a non-pathogenic species by Yasuda et al. (1987). Where as the International Committee on Systemic Bacteriology, Subcommittee on Taxonomy of *Leptospira*, considers L. meyeri serovar ranarum (strain ICF) as pathogenic but L. meyeri serovar semaranga (strain Veldrat Semaranga) as non-pathogenic.

There is an ambiguity PCR product of 630 bp amplified from DNA in urine samples. This product size is not comparable to any PCR products amplified from reference *Leptospires* shown in Table 1. Neither Gravekamp et al. (1993) nor our results on *Leptospira* references gave this ambiguity PCR product. Further investigation is required for isolated Leptospires from that urine sample to confirm the presence of this ambiguity PCR product. The sequencing of this PCR product is necessity as well.

Considering the ambiguity PCR product (a 630 bp) as if derived from unknown pathogenic *Leptospira*, there is totally 17.8% cows detected as carrier and shedder of this pathogen. Although this number is surprisingly low comparing to number of cows with high MAT titer (41% with titer of $\geq$ 1:40) and comparing to number of cows with positive with direct examination by darkfield microscope (88%, as shown in Table 2), the result is accordance to a survey performed by Gregoire et al. (1987). Gregoire et al. (1987) reported that it was 24% of cow kidneys from slaughters found *Leptospira* species. However, there were only 13% kidneys containing lesion of interstitial nephritis. These

suggested that cows with interstitial nephritis shed this organism. The physiological adaptation of this organism to survive in cows is complicated. Cows infected with L. interrogans serovar hardjo shed Leptospires from day 18 to day 95 after infection (Gerritsen et al., 1991) but the period after 95 days was not operated. The shedding of *Leptospira* into urine is intermittent (Heath and Johnson, 1994). This could explain for our 2 times lower percentage of carrier cows compared to direct culture from kidneys as reported by Gregoire et al. (1987).

In order to eliminate carrier cows, we can not rely on result from the direct examination under dark field microscope because this technique is not be able to classify pathogenic Leptospira from other spirochete unless an agglutination test is performed together. We also have cope with diagnosis technique relying solely on MAT titer since the percentage of MAT positive cows in Thai is large, 41% as shown in Table 3, and the sero-specific MAT titer in each cows was not agreed with PCR genospecies grouping performed in this study. However, with the combination of MAT and PCR-based method, we are confident in making decision to eliminate and/or to quarantine those animals. Therefore, as you can see in Table 3, there are 12.2% of cows that have to be eliminated and/or 17.8% of cows that have to be quarantined.

CONCLUSION

There were 17.8% cows detected as carrier of *Leptospira* organism in this study. The genospecies grouping of *Leptospires* detected in bovine urine samples by PCR method did not match with the specific serovar tested by MAT. The combination techniques for *Leptospira* diagnosis enable the Veterinarian officers to assure their decisions. Further investigation on physiological adaptation of cows as a host of this organism must be supported for studying and creating new preventive strategies.

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