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PCR-Based Technique for Detection and Differentiation of Pathogenic and Saprophytic *Leptospira* Species

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Abstract: Leptospirosis is one of the important infectious diseases transmitted by artificial insemination. Although there are methods for the rapid detection of *Leptospira*, but identification of species and serovars appears to be the main problem. In this study, a PCR-based technique using three primers was developed. The primers Lepto1 (F), Lepto2(R) and Lepto3(F) were used in the polymerase chain reaction (PCR) to identify and differentiate pathogenic and saprophytic *Leptospira* species on 2% agarose gels. Genomic DNA of nine *Leptospira* species representing 21 pathogenic and 4 saprophytic serovars was tested. The results showed that two bands of 503 and 409 bp were detected in the PCR products of *L. borgpetersenii*, *L. interrogans, L. kirschneri*, *L. noguchii*, *L. santarosai* and *L. weilii* which are classified into the pathogenic group. In contrast, only the 503 bp band was found in *L. biflexa* which is a saprophytic *Leptospira* species. Due to time and cost effectiveness, as well as the efficiency of the PCR using these three primers, this methodology is a potential alternative to discriminate the two groups of *Leptospira* species in routine diagnosis.

Key words: *Leptospira* spp · *Leptospira* species · PCR · 16S rRNA gene

INTRODUCTION

Leptospirosis is one of the important infectious diseases that causes a fetal infection resulting in stillbirths, abortion, infertility, early embryonic death and leads to economic losses to farmers [1]. This disease can be transmitted by artificial insemination because *Leptospira* species can survive under the freezing temperature used to store semen samples [2]. The standard test for diagnosis of the pathogen is the microscopic agglutination test (MAT) which is laborious and time-consuming [1]. Direct diagnostic method based on culture is difficult due to their slow growth and interference by the rapid growth of other contaminants and so is not used for routine diagnosis [1].

The polymerase chain reaction (PCR) also has been used to detect several micro-organisms [2]. This PCR based technique was applied to detect *Leptospira* species in urine samples of cattle that were experimentally infected with serovar *hardjobovis* [3], in the urine of cattle naturally infected with the pathogen [4] and in variety of samples such as urine, kidney and feces in pinniped population [5]. Moreover, TaqMan PCR technique was applied to differentiate between pathogenic and nonpathogeneic *Leptospira* species [6]. Recently, Real-Time PCR was also used to detect pathogenic *Leptospira* species in clinical materials [7] and in urine of dog [8]. In addition, a nested PCR technique was applied to identify *Leptospira* species using primers that derived from the LipL32 sequence [9, 10]. Kositanon *et al.* [11] used nested PCR with specific primers derived from the 23S rDNA of pathogenic and saprophytic *Leptospira* species to differentiate between pathogenic and saprophytic species, while Bomfim *et al.* [12] used primers from the LipL32 sequence of *Leptospira* species to detect pathogenic *Leptospira* species in urine from naturally infected cattle.

Although PCR-based techniques have been successfully applied to the detection and differentiation of *Leptospira* species, several additional methods are

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required for interpretation of the results. For example, Kositanon *et al.* [11] used 2nd-round PCR for nested amplification while Letocart *et al.* [13] used nonradioactive DNA probes for hybridization to discriminate among the pathogenic *Leptospira* species and Wu *et al.* [14] used single strand conformation polymorphism (SSCP).

This study therefore aimed to develop a technique that can be used for detection and differentiation of pathogenic and saprophytic *Leptospira* species by a simple, one step PCR methodology.

MATERIALS AND METHODS

Samples: Nine *Leptospira* species representing 21 pathogenic and 3 saprophytic serovars (Table 1) used in this study were obtained from the National Institute of Animal Health, Department of Livestock Development, Thailand. The samples were cultivated at 28-30°C in liquid EMJH medium [15].

DNA Isolation: Genomic DNA was extracted from 500 µl of pure culture of reference *Leptospira* species using AquarPure Genomic DNA Isolation kit (BIO-RAD) according to the manufacturer's instructions.

PCR Amplification: Leptospira DNA was amplified using specific primers designed from the 16S rRNA gene of *Leptospira* species, Lepto1(F): 5'GTCAAACGGGTAGCAATACC3' Lepto2(R): 5'GTCCGCCTACACACCCTTTAC3' primers [16]. The PCR reaction was performed as described by Shukla *et al.* [16] with some modifications in a total volume of 20 µl containing 20 ng of DNA, 5 pmole each of forward and reverse primers, 200 µM dNTP, 1.5 mM $MgCl_2$, 50 mM Kcl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton® X-100 and 1 U of *Taq* DNA polymerase (Promega), using My Cycler ™ Thermal Cycler (BIO-RAD). PCR was accomplished by 94°C 2 min for one cycle; 94°C for 2 min, 54°C for 1 min, 72°C for 1 min for 35 cycles and final extension at 72°C for 5 min. The amplified products were separated on 5% denaturing polyacrylamide gels and visualized by silver staining [17]. In addition, primer Lepto3(F): 5'AATACTGGATAGTCCCGAGAGGTC3' was newly designed from a sequence of PCR product. The Lepto3(F) was used together with Lepto1(F) and Lepto2(R) to amplified genomic DNA of tested *Leptospira* species. The amplified products were separated on 2% agarose gels, stained with ethidium bromide and visualized under UV light [18].

Table 1: PCR products of *Leptospira* species using Lepto1(F), Lepto2(R) and Lepto3(F) primers

			16S rRNA gene PCR products	
Leptospira	Species	Serovar	503 bp.	409 bp.
Pathogenic	*L. interrogans*	*autumnalis*	+	+
		bataviae	+	+
		canicola	+	+
		djasiman	+	+
		hebdomadis	+	+
		icterohaemorrhagiae	+	+
		pomona	+	+
		pyrogenes	+	+
		sejroe	+	+
	L. borgpetersenii	*javanica*	+	+
		ballum	+	+
		tarassovi	+	+
	L. santarosai	*shermani*	+	+
		sarmin	+	+
		mini	+	+
	L. kirschneri	*grippotyphosa*	+	+
		cynopteri	+	+
	L. weilii	*celledoni*	+	+
	L. noguchii	*louisiana*	+	+
		panama	+	+
	Leptospira sp	*bratislava*	+	+
Saprophytic	*L. biflexa*	*patoc*	+	-
		andamana	+	-
		maintenon	+	-
	L. meyeri	*ranarum*	+	+

Primer Design: The DNA fragments were excised from 5% denaturing polyacrylamide gels. The gel slices were incubated over night in 20 µl of distilled water at 37°C. The eluted DNA was used as a template in PCR reactions using Lepto1 and Lepto2 primers. The PCR products were separated on 1.5% agarose gel and stained with ethidium bromide. The bands containing DNA fragment were then excised from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN). The purified DNA was sequenced with the specific Lepto2 primer.

The sequencing results of pathogenic and saprophytic *Leptospira* species were aligned by ClustalX (1.81) and blast N with NCBI database. Additional sequences of the 16S rRNA gene of *Leptospira* species from the NCBI Entrez database, AY631876 and AY631893 for *L. biflexa*, EF536983 for *L. interrogans*, AY631894 for *L. interrogans* serovar

icterohaemorrhagiae and Z21630 for *L. borgpetersenii* were also aligned in order to compare with our sequences. Finally, specific primes were designed using Vector NTI 5.0 software.

RESULTS AND DISCUSSION

Genomic DNA of 22 pathogenic and 3 saprophytic serovars were used to amplify with Lepto1(F) and Lepto2(R) primers. The PCR products were separated by electrophoresis on 5% polyacrylamide gels. Different patterns of DNA fragments between pathogenic and saprophytic *Leptospira* species were observed (Figure 1). A 503 bp PCR product was found in pathogenic *Leptospira* species serovars, *autumnalis*, *bataviae*, *canicola*, *djasiman*, *hebdomadis*, *icterohaemorrhagiae*, *pomona*, *pyrogenes* and *sejroe*, In contrast, PCR products of saprophytic *Leptospira* species serovars *patoc andamana* and *maintenon* showed only a band of 493 bp while the PCR products amplified from serovar *ranarum* showed bands of 493 and 503 bp. In addition, a band of approximately 600 bp was observed in *Leptospira* species serovars *andamana* and *maintenon*.

As polyacrylamide gel electrophoresis is not generally suitable for routine work, a new primer combination that could be used to identify and differentiate between pathogenic and saprophytic *Leptospira* species was developed. The 503 bp fragment of pathogenic and the 493 bp fragment of saprophytic *Leptospira* species were sequenced and aligned. Finally, a newly developed primer specific to *Leptospira* species, Lepto3(F) was designed from the sequences of the 16S rRNA genes analyzed (Figure 2). The new primer was initially used with the Lepto2(R) primer to amplify genomic DNA of saprophytic *Leptospira* species serovar *patoc* and pathogenic *Leptospira* species serovar *tarassovi*. The results showed that a PCR product of 409 bp was observed from the pathogenic samples. The presence and absence of the PCR product may be easily misinterpreted as a false negative, therefore a combination of Lepto1(F), Lepto2(R) and Lepto3(F) primers was tested. The results showed that using all three primers together could amplify the genomic DNA of all 21 pathogenic and 4 saprophytic serovars. In addition, different patterns of PCR products between the two groups of *Leptospira* species could also be observed on 2% agarose gels. Two bands of 503 and 409 bp were detected in the pathogenic group, while only the 503 bp band was found in saprophytic *Leptospira* species (Table 1 and Figure 3). However, two bands of 503 and 409 bp were observed in serovar *ranarum* which is classified in saprophytic group. This agrees with the results found in Kositanont *et al*. [11]. The same PCR products were obtained when amplified genomic DNA of serovar *ranarum* and pathogenic group using primers designed from 23S rDNA [11].

PCR based techniques have been applied to the detection and differentiation of *Leptospira* species and several primer pairs specific to leptospires have been developed for PCR detection. These primers were designed based on specific gene targets [19], most frequently the 16S or 23S rRNA genes [16, 20-26].In addition, further techniques have also been applied to PCR based detection. LSSP-PCR (low-stringency single specific primer PCR) was used to identify *Leptospira* species in urine samples of cattle with clinical suspicion [12]. This method however requires a two steps PCR and

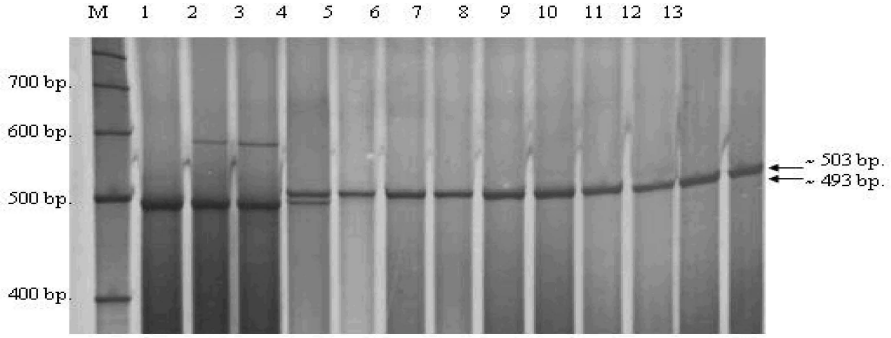

Fig. 1: PCR products of *Leptospira* species using Lepto1/Lepto2 primers on 5% denaturing polyacrylamide gel.
M: 100 bp. + 1.5 kb DNA marker
Lanes 1-4: Saprophytic *Leptospira* species serovars *patoc andamana*, *maintenon* and *ranarum*
Lanes 5-13: Pathogenic *Leptospira* species serovars, *autumnalis*, *bataviae*, *canicola*, *djasiman*, *hebdomadis*, *icterohaemorrhagiae*, *pomona*, *pyrogenes* and *sejroe*
Note: lanes 2 and 3 had addition band at approximately 600 bp, lane 4 had two bands at approximately 500 bp

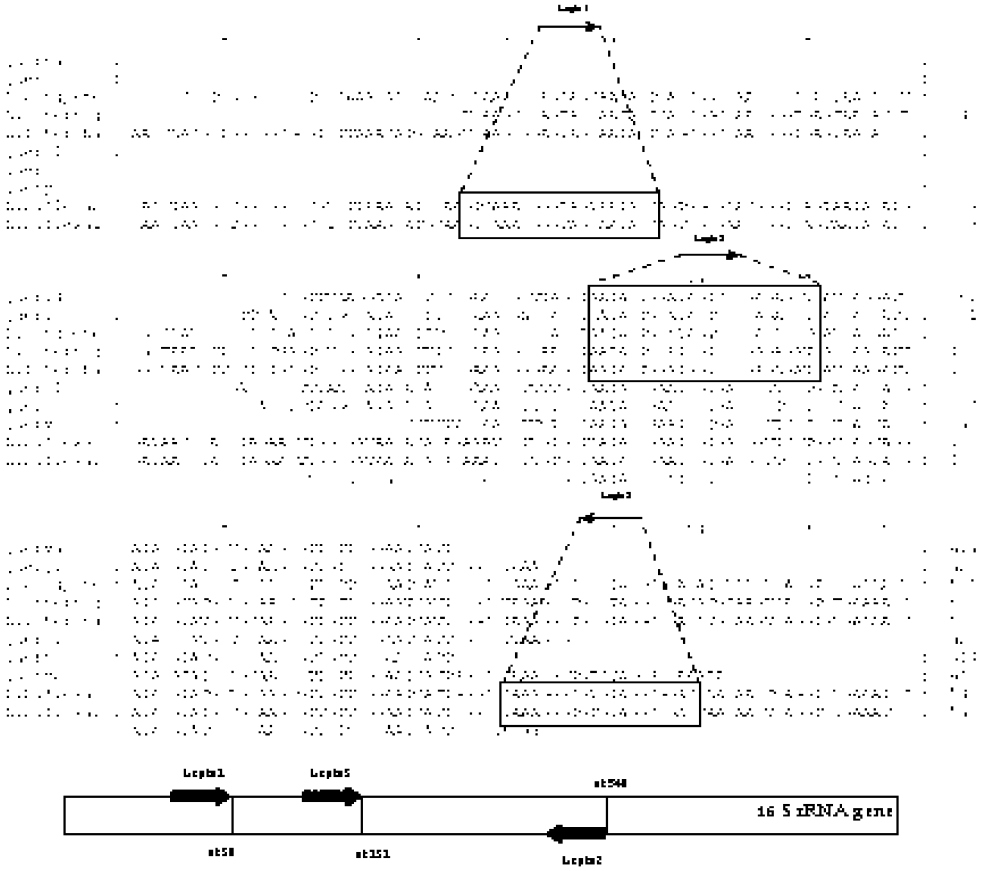

Fig. 2: Sequence alignment of the 16S rRNA gene of *Leptospira* species and the position of Lepto1(F), Lepto2(R) and Lepto3(F) primers
Query1: sequence of 493 bp. fragment of *Leptospira* species serovar *andamana*
Query2: sequence of 493 bp. fragment of *Leptospira* species serovar *maintenon*
Query3: sequence of 493 bp. fragment of *Leptospira* species serovar *patoc*
Query4: sequence of 503 bp. fragment of *Leptospira* species serovar *javanica*
Query5: sequence of 503 bp. fragment of *Leptospira* species serovar *ballum*

analysis of the PCR products on acrylamide gels. Moreover, PCR-RFLP (PCR-restriction fragment length polymorphism) has been applied to detect and differentiate *Leptospira* species in bovine semen [27]. The technique used several restriction enzymes to digest the PCR products followed by analyzed on acrylamide gels. Nested PCR with specific primers derived from 23S rDNA of pathogenic and saprophytic *Leptospira* species has been used to differentiate between pathogenic and saprophytic species [11].

In this study, *L. borgpetersenii*, *L. interrogans, L. kirschneri*, *L. noguchii*, *L. santarosai* and *L. weilii* were classified into the pathogenic group. However, *L. meyeri*, a nonpathogenic *Leptospira* species, was also categorized in the pathogenic group due to the same PCR result obtained. The results in this study are in accordance with Kositanont *et al*. [11] in which *L. meyeri* serovar *ranarum* showed the same PRC products with the pathogenic group. In addition, molecular taxonomic studies using DNA-DNA hybridization that had grouped the pathogenic *Leptospira* species into eight [28] species which were *L. borgpetersenii*, *L. inadai*, *L. interrogans* sensu stricto, *L. kirschneri*, *L. noguchii*, *L. santarosai*, *L. weilii* and *L. meyeri*, Importantly, a simple PCR technique using a primer combination, Lepto1(F), Lepto2(R) and Lepto3(F) primers, has been developed and applied to detect and differentiate pathogenic and saprophytic *Leptospira* species on agarose gels which can be applicable to routine diagnosis.

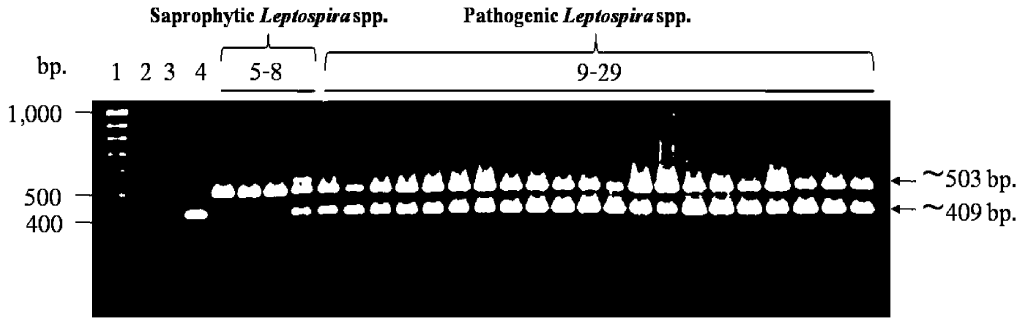

Fig. 3: PCR products of *Leptospira* species using Lepto1(F)/Lepto2(R)/Lepto3(F) and Lepto3(F)/Lepto2(R) primers on 2% agarose gel
Lane 1: 100 bp + 1.5 kb DNA marker
Lane 2: Negative control of multiplex PCR using Lepto1/2/3 primers
Lane 3: Saprophytic Leptospira serovar *patoc* using Lepto 2/3 primers
Lane 4: Pathogenic Leptospira serovar *tarassovi* using Lepto 2/3 primers
Lanes 5-8: Saprophytic Leptospira (*L. biflexa* serovars *patoc andamana* and *maintenon*; and *L. meyeri* serovar *ranarum*) using Lepto1/2/3 primers
lane 9-29: Pathogenic *Leptospira* species (*L. interrogans* serovars *autumnalis*, *bataviae*, *canicola*, *djasiman*, *hebdomadis*, *icterohaemorrhagiae*, *pomona*, *pyrogenes* and *sejroe*; *L. borgpetersenii*s serovars *javanica*, *ballum* and *tarassovi*; *L. santrosai* serovars *shermani*, *sarmin* and *mini*; *L. kirschneri* serovars *grippotyphosa* and *cynopteri*; *L. weilii* serovar *celledoni*; *L. noguchii* serovars *louisiana* and *panama*; and *Leptospira* species serovar *bratislava*) using Lepto1/2/3 primers

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