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Detection of pathogenic leptospires in animals by PCR based on *lipL21* and *lipL32* genes

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Efficacy of primers capable of amplifying conserved outer membrane protein (OMP) genes i.e., *lipL21* and *lipL32* of *Leptospira* strains was tested for rapid and early diagnosis of the leptospirosis using a polymerase chain reaction (PCR). These OMP genes were found to be conserved in various leptospiral serovars viz., Canicola, Pomona, Icterohaemorrhagiae, Pyrogenes, Sejroe, Grippotyphosa, Ballum and Tarassovi as PCR products of 561 bp and 756 bp were obtained by PCR employing *lipL21* and *lipL32* based primers, respectively, in all these serovars. Absence of such amplicons in DNA extracted from *Pasteurella, Campylobacter* and *Brucella* confirmed the specificity of the primers. Serum and tissue samples collected from cattle, buffaloes and experimentally infected guinea pigs and calves were subjected to PCR using above primers as well as conventionally used primers G1/G2. All the sera and tissue samples, whether field samples or collected from experimentally infected animals, found positive for G1/G2 specific PCR were also positive for *lipL21* and *lipL32* specific PCR. The present study indicated that *lipL21* and *lipL32* based primers could be used for PCR based diagnosis of leptospirosis. Since G1/G2 primers are known not to amplify the DNA of Grippotyphosa, the use of primers employed in the present study could have an additional advantage in detection of cases of the disease.

Keywords: *Leptospira*, Outer membrane proteins, Polymerase chain reaction

Leptospirosis is an economically important disease affecting most of domestic animals including cattle, buffalo, dog, sheep, goat and horse along with wild animals. It is characterized by abortion, stillbirth, infertility, decreased milk production and death in domestic animals[1]. As the course of leptospirosis varies from mild to rapidly fatal forms, the laboratory based techniques are important for arriving at the definite diagnosis. Culturing of the organism is most demonstrative approach, but this technique is very laborious and time consuming[2]. Serological techniques to access the specific antibody response do not contribute to early diagnosis of the disease as it becomes detectable after a period of 7-10 days of the onset of illness.

Polymerase chain reaction (PCR) based molecular techniques can be potentially rapid and specific means of diagnosis of leptospirosis especially in case of outbreaks. PCR has been used for the diagnosis of fastidious organisms[3]. As regards leptospirosis, several researchers have used PCR to detect the *Leptospira* species in a variety of clinical specimens[4-10]. In the present study, efficacy of primers capable of amplifying conserved outer membrane protein (OMP) genes i.e., *lipL21* and *lipL32* was tested using serum and tissue samples collected from cattle, buffaloes and experimentally infected guinea pigs. These genes were targeted as they are reported to be present in all the pathogenic *Leptospira* serovars and possess extensive sequence homology[11-13].

Materials and Methods

Bacterial strains and media—The *Leptospira* serovars used in the present study were—*L. interrogans* serovars Canicola, Pomona, Icterohaemorrhagiae, Pyrogenes and Sejroe, *L. kirschneri* serovar Grippotyphosa, *L. borgpetersenii* serovars Ballum and Tarassovi. The cultures were maintained at 28°-30°C in liquid EMJH (Ellinghausen McCullough Johnson Harris)[14] medium by routine subculture at 7 to 10 day intervals. The semisolid EMJH medium with agar (0.2%) was used for

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maintenance of stock cultures. In addition, the cultures of *Pasteurella, Campylobacter* and *Brucella* were also included in the study to test the specificity of primers used in PCR.

Genomic DNA isolation—The genomic DNA from *Leptospira* serovars were isolated by CTAB method[15] and checked for purity using 1.5% agarose gel. It was used as template in PCR assay using primers based on *lipL21* and *lipL32* genes. Additionally, DNA from *Pasteurella, Campylobacter* and *Brucella* were also purified for testing specificity of the PCR.

Experimental animals—A total of 8 day old guinea pigs were given intraperitoneally 1 ml of 5 days old Canicola culture (2×10^8 leptospires/ml). Tissue and serum samples were collected from the dead animals and subjected to PCR. Additionally, four buffalo calves, each was given subcutaneously 3-5 ml of the Canicola culture and serum samples from these animals were subjected to PCR examination.

Test samples—A total of 60 kidney and 45 liver samples were collected from buffaloes slaughtered at a local abattoir and labeled as K1 to K60 and L1 to L45 for kidney and liver samples, respectively. Additionally, serum samples (50) were collected from cattle with a history of reproductive disorders from various states of the country such as Uttaranchal, Tamil Nadu, Orissa etc. and were labeled as S1 to S50. These samples were preserved at -20°C till used for PCR assay. All these samples were subjected to PCR based on *lipL21, lipL32* as well as G1/G2 primers.

Sample preparation—The serum samples were prepared for PCR as per the method of Merien *et al.*[16] with slight modifications. Two ml of the suitably diluted samples were centrifuged at 10,000 rpm for 30 min and the pellet was resuspended in 0.5 ml distilled water. Thereafter, the suspension was heated to 96°C for 10 min and kept on ice to inactivate any residual enzymatic activity. Tissue samples (1-2 g each) were triturated in 2-3 ml PBS (*p*H 7.2) and centrifuged at 6,000 rpm for 15 min to remove the coarse particles and tissue debris. The supernatant was used for DNA extraction by CTAB method[15].

Oligonucleotides—PCR was performed using three sets of primers, one being the conventional i.e., G1/G2[5] and the *lipL21* and *lipL32* based primers under test. Primers G1 and G2 had the sequences 5′CTG AAT CGC TGT ATA AAA GT 3′ and 5′GGA AAA CAA ATG GTC GGA AG 3′, respectively. The primers for amplification of *lipL21* gene were designed from the previously reported gene sequence of *L. kirschneri* serovar Grippotyphosa[12]. The forward and reverse primers had sequences of 5' CGC GGT CGA CAT GAT CAA TAG ACT TAT AGC TC3' and 5’CGC GCT GCA GTT ATT GTT TGG AAA CCT CTT G 3', respectively. The primers for amplification of *lipL32* gene were also designed from the previously reported gene sequence of *L. kirschneri* serovar Grippotyphosa[11]. The forward and reverse primers had sequences of 5' GTC GAC ATG AAA AAA CTT TCG ATT TTG 3' and 5’CTG CAG TTA CTT AGT CGC GTC AGA AGC 3', respectively. Aliquots of these primers were prepared in nuclease free water (Sigma) to achieve final concentration of 25 pmol/µl.

PCR assay—The PCR was conducted using a 25 µl reaction mixture that consisted of 2.5 µl of 10 × PCR buffer, 1 µl of 10 m*M* dNTP mix, 1.5 µl of 25 m*M* $MgCl_2$, 1.0 U of *Taq* DNA polymerase, 1 µl (25 pmol) each of forward and reverse primer and 50 ng of template DNA. *lipL21* and *lipL32* specific PCR was performed with the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 sec, extension at 72°C for 30 sec and a final extension at 72°C for 6 min. The conditions for G1/G2 specific PCR were same except for the annealing (1 min) and extension (2 min) time. The products were checked for amplification and absence of spurious products by electrophoresis using 1.5% agarose gel. The gel was visualized by gel documentation system and photographed.

Results

DNA from all the *Leptospira* serovars when subjected to PCR amplification, amplicons of 561 and 756 were observed using *lipL21* and *lipL32* specific primers, respectively. No such amplicons were observed using DNA extracted from *Pasteurella, Campylobacter* and *Brucella.*

Infected guinea pigs and calves—Using all three sets of primers, PCR amplified the leptospiral DNA in 5 out of 8 serum samples collected from guinea pigs within 72 h post infection. No amplification was observed in samples collected beyond this period. Of eight guinea pigs, three died between 24 to 48 h after the infection and liver samples from all three were found PCR positive. Five animals survived the infection and were sacrificed on day-7 post infection. Liver samples from these sacrificed animals were

found negative by PCR (Table 1). Fig. 1 depicts the gene of 285 bp, amplified from serum and liver tissue using G1/G2 primers. The amplification achieved with *lipL21* and *lipL32* has been shown in Figs 2 and 3, respectively. As regards infected calves, serum samples from all 4 calves, collected within 5 days post infection were found to be positive for PCR using all three sets of primers. Figs 4 and 5 depict the amplified *lipL21* and *lipL32*, respectively, from only a few of the representative samples.

Field samples—A total of 4 (S8, S23, S36 and S49) out of 50 serum samples from cattle and 2 (L19 and L21) out of 45 liver samples from buffaloes were found positive in *lipL21* based PCR. The same samples were also detected positive using *lipL32* as well as G1/G2 primers. As regards kidney samples from buffaloes, 5 (K2, K10, K25, K26 and K43) out of 60 were positive using *lipL21* specific primers. These positive samples were also detected by *lipL32* based PCR, whereas G1/G2 primers could detect only 4 of these (K26 was found negative). In positive samples, the amplified products of 285 bp, 561 bp, and 756 bp were obtained using G1/G2, *lipL21* and *lipL32* based primers, respectively (Figs 6-8). A comparison of PCR results of serum, liver and kidney samples employing above three sets of primers is given in Table 2.

Table 1—PCR conducted on serum and tissue samples of guinea pigs experimentally infected with *Leptospira interrogans* serovar Canicola

	Serum samples	Liver samples	Kidney samples
Total number of samples	8	8	8
G1/G2 specific PCR positive	5	3	0
lipL32 based PCR positive	5	3	0
lipL21 based PCR positive	5	3	0

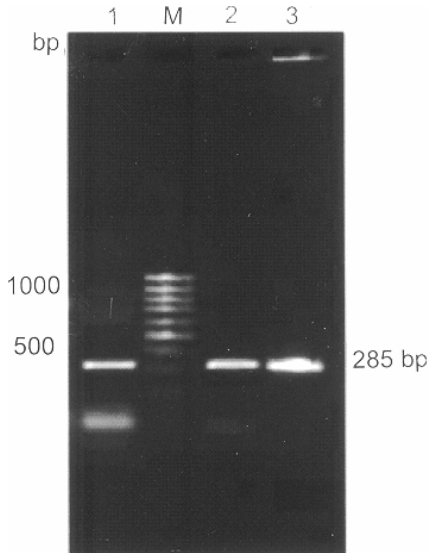

Fig. 1—Amplification of 285 bp DNA fragment by G1/G2 specific PCR from serum and liver samples of representative experimentally infected guinea pigs. [Lane M, 100 bp DNA ladder; Lane 1, Canicola DNA taken as control positive; Lane 2, serum sample; Lane 3, liver sample]

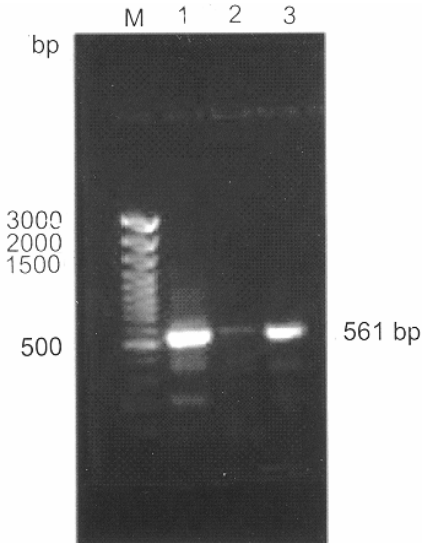

Fig. 2—PCR amplification of *lipL21* gene from serum and liver samples of representative experimentally infected guinea pigs. [Lane M, 100 bp DNA ladder; Lane 1, Canicola DNA taken as control positive; Lane 2, serum sample; Lane 3, liver sample]

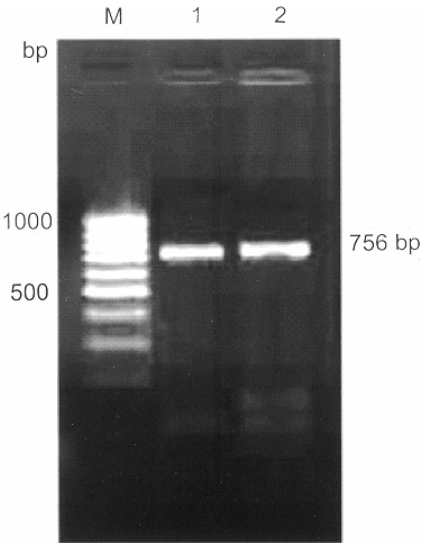

Fig. 3—PCR amplification of *lipL32* gene from serum and liver samples of representative experimentally infected guinea pigs. [Lane M, 100 bp DNA ladder; Lane 1, serum sample; Lane 2, liver sample]

Discussion

One of the basis for using *lipL21* and *lipL32* gene based primers for diagnosis of leptospirosis was that these primers target a known gene sequence reported to be conserved in all the pathogenic *Leptospira* serovars[11-13]. However, before screening the samples by PCR, the conserved nature of *lipL21* and *lipL32* OMP genes in leptospiral serovars viz., *Leptospira interrogans* serovars Canicola, Pomona, Icterohaemorrhagiae, Pyrogenes and Sejroe, *L. kirschneri* serovar Grippotyphosa, *L. borgpetessenii* serovars Ballum and Tarassovi was confirmed. In all these serovars, the PCR product of 561 bp and 756 bp were obtained employing *lipL21* and *lipL32* based primers, respectively. Further, the absence of such amplicons in PCR employed on DNA

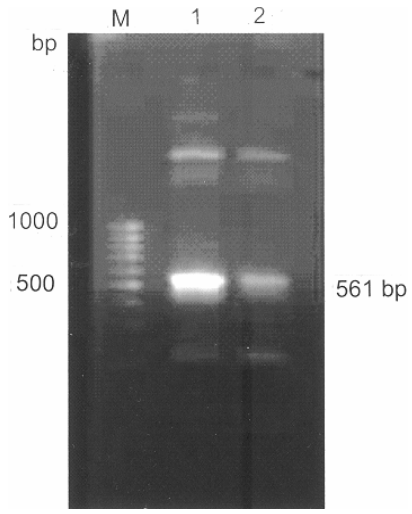

Fig. 4—PCR amplification of *lipL21* gene from serum samples of representative experimentally infected calves. [Lane M, 100 bp DNA ladder; Lane 1 and 2, serum samples from calves]

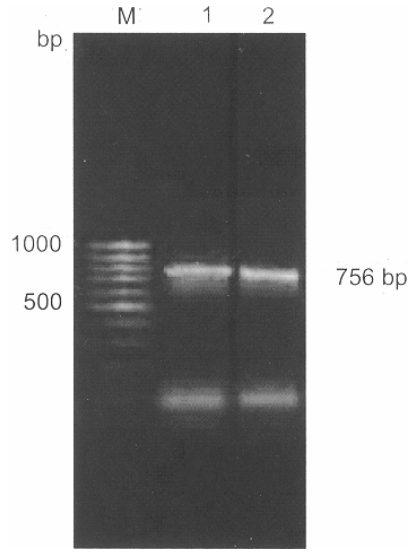

Fig. 5—PCR amplification of *lipL32* gene from serum samples of representative experimentally infected calves. [Lane M, 100 bp DNA ladder; Lane 1 and 2, serum samples from calves]

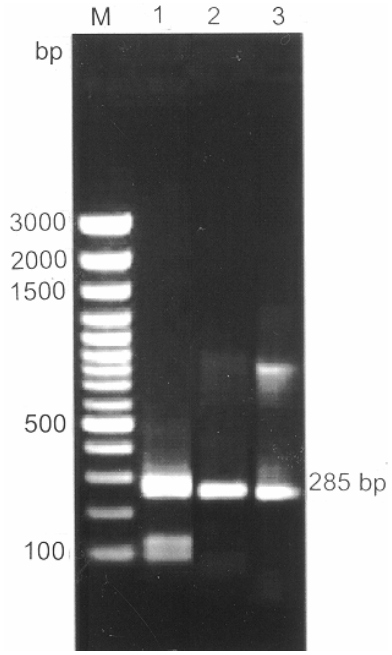

Fig. 6—Amplification of 285 bp DNA fragment by G1/G2 specific PCR from field samples. [Lane M, 100 bp DNA ladder; Lane 1, serum sample (S23) from cattle; Lanes 2 and 3, liver (L19) and kidney (K10) samples, respectively, from buffaloes]

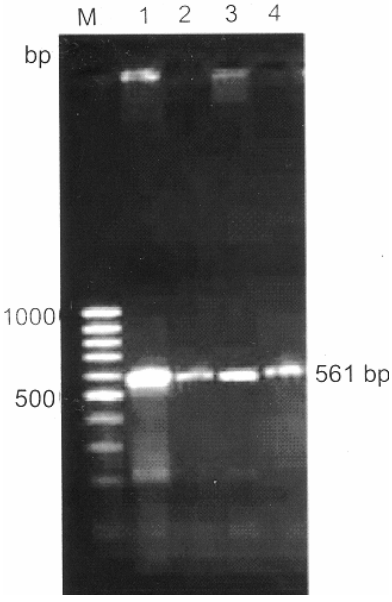

Fig. 7—Amplification of *lipL21* gene by PCR from field samples. [Lane M, 100 bp DNA ladder; Lane 1, Canicola DNA taken as control positive; Lane 2, serum sample (S23) from cattle; Lanes 3 and 4, liver (L19) and kidney (K10) samples, respectively, from buffaloes]

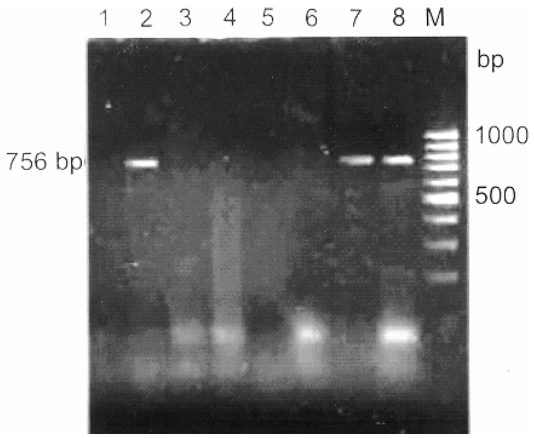

Fig. 8—Amplification of *lipL32* gene by PCR from field samples. [Lane M, 100 bp DNA ladder; Lanes 1 and 2, serum samples (S15 and S23, respectively) from cattle; Lanes 3, 4 and 7, liver samples (L6, L13 and L19, respectively) from buffaloes; Lanes 5, 6 and 8, kidney samples (K3, K36 and K10, respectively) from buffaloes]

Table 2—Detection of leptospires in samples collected from cattle and buffaloes using PCR

	Cattle serum samples		Buffaloes	
	Experi-mentally infected	Field	Kidney samples	Liver samples
Total number of samples	4	50	60	45
G1/G2 specific PCR positive	4	4	4	2
lipL32 based PCR positive	4	4	5	2
lipL21 based PCR positive	4	4	5	2

extracted from *Pasteurella, Campylobacter* and *Brucella* confirmed the specificity of the primers.

PCR was tested for its efficacy using serum and tissue samples of guinea pigs and calves experimentally infected with *L. interrogans* serovar Canicola. A total of 5 out of 8 guinea pig serum samples were found positive by PCR followed by liver samples (3 out of 8), whereas none using the kidney samples. It is obvious because in guinea pigs a very acute form of the disease is produced, characterized by the presence of organism in blood, liver and spleen etc. The organisms have a tendency to lodge in kidneys when the acute phase is over[2]. In the present study, kidney samples collected on day 7 from the sacrificed guinea pigs, which survived the infection, were also negative indicating that the lodging had not occurred.

When PCR was applied on infected calf sera, amplification was observed using all three sets of primers, but this was observed in sera collected within five days post inoculation indicative of the occurrence of leptospiraemia. None of these animals died during the examination period. Hence, examination of the tissues for possible lodging of leptospires could not be done.

Application of PCR for detection of leptospires in various field animals was also studied. As regards the field samples, 4 of 50 cattle sera and 2 of 45 liver samples from buffaloes were positive using all three sets of primers (Table 2). Among 60 kidney samples, 5 were detected by *lipL21* and *lipL32* specific PCR, whereas only 4 of these 5 could be detected by G1/G2 specific PCR. In the present study, G1/G2 specific PCR was taken as standard and results of *lipL21* and *lipL32* specific PCR were compared with it. The results depicted that all the sera and tissue samples, whether field samples or collected from experimentally infected animals, positive for G1/G2 specific PCR were also positive for *lipL21* and *lipL32* specific PCR. Additionally, one kidney sample from buffalo, negative for G1/G2 PCR was found positive in *lipL21* and *lipL32* specific PCR. Variation in results of only one sample may not lead to any conclusion on the superiority of one PCR over the other. The results of the present study were, therefore, suggestive of the similarity in the efficacy of all three sets of primers for conducting PCR; however, further study is needed to authenticate the relative efficacy of *lipL21* and *lipL32* specific PCR employing a large number of samples collected from varied sources.

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References

1 Haake D A, Martinich A, Summers T A, Shang E S, Pruetz J D, McCoy A M, Mazel M K & Bolin C A, Characterization of leptospiral outer membrane lipoprotein LipL36: Downregulation associated with late log-phase growth and mammalian infection, *Infect Immun*, 66 (1998) 1579.

2 Faine S, *Guidelines for the control of leptospirosis*, Offset Publication No 67, (World Health Organization, Geneva), 1982.

3 Eaglesome M D & Garcia M M, Microbial agents associated with bovine genital tract infection and semen, Part I - *Brucella abortus, Leptospira, Campylobacter fetus* and *Trichomonas foetus*, *Vet Bull*, 62 (1992) 743.

4 Van Eys G J J M, Zaal J, Schoone G J & Terpstra W J, DNA hybridization with Hardjo bovis-specific recombinant probes as a method for Type discrimination of *Leptospira interrogans* Serovar *hardjo*, *J Gen Microbiol*, 134 (1988) 567.
5 Gravekamp C, Van der Kemp H, Franzen M, Carrington D & Schoone G J, Detection of seven species of pathogenic leptospires by PCR using two sets of primer, *J Gen Microbiol*, 139 (1993) 1691.
6 Heinemann M B, Garcia J F, Nunes C M, Morais Z M, Gregori F, Cortez A, Vasconcellos S A, Visintin J A & Richtzenhain L J, Detection of leptospires in bovine semen by polymerase chain reaction, *Aust Vet J*, 77 (1999) 32.
7 Heinemann M B, Garcia J F, Nunes C M, Gregori F, Higa Z M M, Vasconcellos S A & Richtzenhain L J, Detection and differentiation of *Leptospira* spp. Serovars in bovine semen by polymerase chain reaction and restriction fragment length polymorphism, *Vet Microbiol*, 73 (2000) 261.
8 Senthil Kumar A, Ramadass P & Nachimuthu K, Use of polymerase chain reaction for the detection of the leptospires in clinical samples, *Indian Vet J*, 78 (2001) 1087.
9 Harkin K R, Roshto Y M & Sullvan J T, Clinical application of polymerase reaction assay for diagnosis of leptospirosis in dogs, *J Am Vet Med Assoc*, 222 (2003) 1224.
10 Oliveira M A, Cabellero O L, Vago A R, Hartskeerl R A, Romnah A J, Pena S D, Simpson A J & Kaury M C, Low-stringency single specific primer PCR for identification of *Leptospira*, *J Med Microbiol*, 52 (2003) 127.
11 Haake D A, Chao G, Zuerner R L, Barnett J K, Barnett D, Mazel M, Matsunaga J, Levett P N & Bolin C A, The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection, *Infect Immun*, 68 (2000) 2276.
12 Cullen P A, Haake D A, Bulach D M, Zuerner R L & Adler B, LipL21 is a novel surface exposed lipoprotein of pathogenic Leptospira species, *Infect Immun*, 71 (2003) 2414.
13 Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Reis M G, Levett P N, Ko A I & Haake D A, Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans, *Infect Immun*, 69 (2001) 4958.
14 Johnson R C, Walby J, Henry R A & Auran N E, Cultivation of parasitic leptospires: Effect of pyruvate, *Appl Microbiol*, 26 (1973) 118.
15 Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A & Struhl K, Current Protocols in Molecular Biology, in *Current protocols* Vol I (John Wiley and Sons, Inc, USA) 1995, 2.4.1.
16 Merien F, Amouriaux P, Perolat P, Baranton G & Saint Girons I, Polymerase chain reaction for detection of *Leptospira* spp in clinical samples, *J Clin Micro*, 30 (1992) 2219.