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16S r RNA PCR FOR CHARACTERIZATION OF LEPTOSPIRA FROM ANDHRA PRADESH

***Dr. D. Rani Prameela[1], Dr. D. Sreenivasulu[2], Dr. N. Nataraj Seenivasan[3], Dr. P. Vijayachari[4] and S. Vijaya Lakshmi[5]**

[1,2 and 5]Department of Veterinary Microbiology, College of Veterinary Science
Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh
[3]Professor, Department of Microbiology, Trichy, Bharathy Darshan University
[4]Regional Medical Research Station, Port Blair-744 001, Andaman and Nicobar Islands
Email: raniprameela.dr@gmail.com (**Corresponding Author*)

Abstract: A total of 458 samples were collected from clinically suspected cases and processed for the isolation of Leptospira using EMJH liquid medium. A total of 17 leptospiral isolates, 5 from sheep (42), 4 from pigs (15), 2 from humans (53), one from rice field water(10) and 5 from rodents (299)were recovered from suspected samples.

The 17 leptospiral isolates were purified using 0.22 µM membrane filters. The isolates were further subjected to pathogenic studies using Guinea pigs. The organisms were re-isolated and maintained in the laboratory. Molecular characterization of the isolates was studied using 16SrRNA PCR using specific primers for the identification of pathogenic leptospira. The selected amplified PCR products of the leptospiral isolates from different species were sent for sequencing analysis. Based on DNA sequencing results of 16SrRNA, the isolates recovered from sheep were identified as *Leptonema illini* (S_1), *L. hardjo* (S_3), *L. inadai* (S_4). Among rats, *L.noguchi* (RR1) and *Leptonema illini* (RG1) and from pigs, *L. Pomona* (P4).

Keywords: EMJH liquid medium, Purification, Pathogenic studies, Molecular Characterisation, 16 SrRNA PCR and sequence results.

Introduction:

Leptospirosis is considered as most wide spread zoonotic disease in the world (Turner 1970) affects humans and wide variety of animals (Vinetz 2001). The diagnosis of Leptospirosis is difficult because the signs are non specific and confusing with other diseases. The disease usually diagnosed based on the demonstration of serum antibodies by microscopic agglutination test (MAT), enzyme linked immunosorbent assay (ELISA) or isolation of leptospires from infected animals. Low sensitivity of MAT during the early stage of the disease, requirement of paired sera samples for correct interpretation of the test are the major constraints to carry out the MAT. Ig M ELISA found to have high sensitivity but the limitation is that IG M antibodies persist in sera of infected humans for long time, with out clinical disease (Silva et al 1997). Recent years the introduction of molecular techniques such

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as Southern blot analysis (Van eyas et al 1988 & 1991; Zuerner and Bolin 1990; Perolat et al 1990 and Pacciarini et al 1992). Pulsed field gel electrophoresis (Herrmann et al 1991 & 1992), dot blot and in-situ hybridization analysis (Miller et al 1987; Terpestra et al 1987 & Zuerner and Bolin 1988) helped in over coming the limitations of traditional serological techniques.

The phenotype tests currently used are unable to distinguish between species of leptospira and often don't reflect the genetic relatedness (Levett 2001; Bharati et al 2003 and Vijayachari et al 2003). The difficulties associated with the tests led to the development of molecular methods for identification and sub-typing of leptospira. Hence in the present study PCR was standardized for characterization of leptospires targeting 16s rRNA gene.

Material and Methods:

Collection of samples:

Samples were collected during the period from February 2006 to May 2010. A total of 159 samples from clinically suspected cases of Cattle (26), Sheep (42), Dogs (13), Pigs (15), Humans (53), stagnated water in Rice fields of outbreak area (10). A 10% homogenous suspension of kidneys and livers collected from 299 Rats trapped from different geographical areas. **(Table.**1)

Preparation of EMJH medium:

EMJH liquid medium with Tween 80, Antibiotics and 5-fluorouracil was used as a selective medium according to Johnson and Harris, 1967 with slight modifications.

Isolation of *Leptospira*:

All the clinical samples collected were processed and inoculated into EMJH liquid media and incubated at 29^{o}C ± 1^{o}C in B.O.D. incubator and screened for presence of *Leptopira* at weekly intervals under dark-field microscope.

Purification of *Leptospiral* isolates:

Filtration: Leptospiral isolates obtained from different sources were purified by filtration through Cellulose membrane filters with 0.22µm. (M/S Sartorius, India) and cultured freshly into EMJH media.

Animal inoculation: Pathogenic studies were carried out in Guinea Pigs weighing 150 to 250 gms. (I/P). 12 hours of post inoculation 0.5 ml. of heart blood was inoculated into EMJH media for re-isolation.

Molecular Characterization

DNA Extraction from cultures

DNA was extracted according to the method of Boom et al 1990. 4 ml of well grown leptospiral cultures were centrifuged at 13,000rpm for 15min. The pellet was washed twice and re-suspend in 500 µl of solution I (Appendix I). Later 50 µl of lysozyme (5mg/ml dissolved in solution I) was added and incubated at 37^{O}C for 15 min. Later added 50 µl of 10percentage SDS, 5 µl of proteinase K (10mg/ml) and incubated at 65^{O}C for 30 min. After that 40 µl of 5M Nacl and 32 µl of CTAB Nacl were added and incubated at 65^{O}C for 30min. Later equal volumes of chloroform isoamyl alcohol (241) (approximately 677 µl) was added. Then the contents were vortexed and centrifuge at 13,000 rpm for 15min. The supernatant (300 µl approximately) was collected and 180 µl of chilled ethanol was added. After gentle mixing the contents were kept at -70^{O}C for 3hrs. Finally centrifugation was done at 10,000 rpm for 30min to pellet down the DNA. Contents were decanted and the pellet was dried over night and reconstituted in 50 µl of TE buffer and stored at -20^{O}C until used.

PCR to amplify 16S r RNA:

16S r RNA amplification was carried out according to the method of Shukla et al 2003 with certain modifications.

S.No	Primer used	Sequence
1.	FP	5’ GGC GGC GCG TCT TAA ACA TG 3’
2.	RP	5’ GTC CGC CTA CGC ACC CTT TAC G 3’

In brief PCR was carried out in a gradient thermal cycler (eppendorf) for 30 cycles using a set of specific primers I & II using following steps. Initial denaturation at 94^{0}C for 5 min, annealing at 63^{0}C for 45 sec, extension at 72^{0}C for 1 min followed by cyclic denaturation at 94^{0}C for 1 min, final annealing at 63^{0}C for 15 minutes and final extension for 15 min at 72^{0}C. Each 50µl of reaction mixture consisted of 25mM MgCl2, 200mM dNTPs, 10mM Tris HCL, 50mM KCL, 0.5U of Taq DNA polymerase, 20 Pico moles each of primer and 2µl of template DNA. The amplified products were analyzed in 1 percent agarose gel electrophoresis and stained with ethidium bromide. The amplified products were visualized under UV transilluminator and documented using gel doc system (alpha innotech, Alpha Imager HP system). The size of the expected amplified product was 525bp and 1kb DNA ladder (Sigma Aldrich) was used.

h) DNA sequence analysis:

Amplified PCR products of 16 S r RNA was sent to M/s. Bio-serve biotechnologies Pvt.Ltd, Hyderabad for sequencing. The results of the sequence was subjected to nucleotide sequence analysis and homology study using BLAST N search (Clustal W2)

Results:

16SrRNA amplification

The DNA extracted from all the 17 lepospiral isolates were subjected to 16S rRNA amplification. The results are shown in the (**Table. 2** and **Fig.1**). The primer 1 and Primer 2 used in the study amplified the expected gene and yielded 525bp product which is specific to leptospira from all the isolates.

Sequence of DNA analysis of selected amplicons of leptospira organism recovered from Bioserve Pvt. Ltd, Hyderabad (**Fig.2**), was subjected to BLAST analysis and results are shown in the **Table 3**. Results of the study indicated that the isolate S3 recovered from sheep showing 90.5 percent homology with *L.hardjo*, the reference strain. Further the isolate P4 recovered form pigs showed the homology of 92 percent with *L.Pomona* reference strain. One of the isolate S1 obtained from the sheep found to have 100 percent homology with *Leptonema illini*, and the isolate S4 from sheep found to have 100 percent homology with *Leptospira inadai* Similarly, the isolate RR1 obtained from rat found to have 93 percent homology with *L.noguchi* serovar panama strain and RG1 obtained from kidney sample of rat found to have 98 percent homology with *Leptonema.illini*.

Discussion:

A total of 17 leptospira isolates received from sheep (5), pigs (4), rats (5), humans (2) and rice field water (1) were maintained in the laboratory used for characterization. Characterization of leptospiral isolates is an important tool for identification of leptospiral strains. Molecular characterization of leptospiral isolates was studied using 16S rRNA PCR. Leptospiral isolates were subjected for 16S rRNA PCR amplification for specific identification of leptospires by amplification of conserved gene of 16S rRNA using specific primers (Primer I and Primer II) for identification of pathogenic leptospires. The selected amplified PCR products of the leptospiral isolates from different species were sent for sequencing analysis (Bioserve, Hyderabad). The DNA sequencing analysis of sheep isolate S1 showed 100.00 percent homology with *leptonema* illini, S3 had 90.5 percent homology with *L.hardjo* reference strain. S4 found to have 100.00 percent homology with *L.inadai.* Among rat isolates RR1 had 93.00 percent homology with *L.noguchii* strain panama. RG1

isolate showed 98.00 percent homology with *leptonema* illini, pig isolate P4 had 92.00 percent homology with *L. pomona.* 16S rRNA is simple and specific method of identification of pathogenic leptospires from different species. Several workers used 16S rRNA for identification of pathogenic leptospires (Merien et al 1992; Shukla et al 2003; Turk et al 2003 and Wangroongsarb 2005 and Djadid et al 2009). This was first isolation and characterization of leptospiral isolates from Andhra Pradesh. By this study commonly circulating serovars like *L.hardjo*, *L. pomona* and *L.noguchi* were identified, and other serovars which was not familiar like *L.inadai* was also isolated and identified. Apart from this a new genome species *leptonema* was also identified. Though *leptonema* considered as non parasitic (Teresa et al 1993; Hlavata and Bazovska 1974 and Gangadhar Rao et al 2005) reported the pathogenic role of leptonema in animals.

Conclusion

A total of 458 clinical samples were collected from different species cattle (26)sheep(42) dogs (13), pigs(15), humans (53), stagnated water in rice fields (10) and rodents (299) from different geographical areas were collected and processed for isolation. EMJH liquid medium used for isolation of Leptospira. A total o 17 leptospiral isolates 5 from sheep, 4 from pigs, 2 from humans, one from rice field water and 5 from rodents were recovered from suspected samples. All the isolates were purified and maintained in the EMJH semisolid agar medium. Initially the leptospiral isolates subjected for physico chemical characterization and later for molecular characterization using 16SrRNA PCR. On DNA sequencing analysis, the isolates from sheep were identified as *Leptonema illini* (S_1), *L. hardjo* (S_3), *L. inadai* (S_4). Among rats, *L.noguchi* (RR1) and *Leptonema illini* (RG1) and from pigs, *L. Pomona* (P4).

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References

[1] Bharati, A., Nally, J., Ricaldi, J., Matthias, R., Willing, M., Gotuzzo, E. and Vinetz, J. (2003). *Leptospirosis*: A zoonotic disease of global importance. Lancet infectious diseases. Vol 3:757-771.

[2] Boom, R.C., Sol, J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-Van-Dillen , P.M.E. and Noornaa, J.V.D. (1990). Rapid and simple method for purification of Nucleic acids. Journal of Clinical Microbiology. Vol 28: 495-503.

[3] Corney, B.G., Colley, J., Djordjevic, S.P., Whittington, R. and Graham, G.C. (1993). Rapid identification of some *Leptospira* isolates from cattle by random amplified polymorphic DNA fingerprinting. Journal Clinical Microbiology. Vol 31: 2927–2932.

[4] Djadid, N.D., Ganjia, Z.F., Gouyab, M.M., Rezvanic, M. and Zakeria, S. (2009). A simple and rapid nested polymerase chain reaction-restriction fragment length polymorphism technique for differentiation of pathogenic and nonpathogenic *Laptospira* spp. Diagnostic Microbiology and Infectious Disease. Vol 63: 251-256.

[5] Gangadhar, N.L., Rajasekhar, M. and Prabhudas, K. (2005). Isolation of leptonema from rats and cattle in India. Indians Journal of Animal Science. Vol 75:508-510.

[6] Herrmann, J.L., Baril ,C., Bellenger, E., Perolat, P., Baranton, G. and Saint Girons, I. (1991). Genome conservation in isolates of *Leptospira interrogans*. Journal of Bacteriology. Vol 173:7582–7588.

[7] Herrmann, J.L., Bellenger, E., Perolat, P., Baranton, G. and Saint Girons, I. (1992). Pulsed-field gel electrophoresis of *Not*I digests of leptospiral DNA: anew rapid method of serovar identification. Journal of Clinical Microbiology. Vol 30:1696–1702.

[8] Hlavatz and Bazobska, S. (1974). The possibility of differentiation of pathogenic and saprophytic strains of leptospires by their survival in organisms of laboratory animals. Folia faculty of Medicine Comenianea, Bratislava. Vol 12:163-169.

[9] Johnson, R.C. and Harris, V.G. (1967) Differentiation of pathogenic and saprophytic leptospires, growth at low temperatures. Journal of Bacteriology. Vol 94:27-31.

[10] Johnson, R.C. and Rogers, P. (1964). 5-fluorouracil as a selective agent for growth of leptospirae. Journal of Bacteriology. Vol 87:422-426.

[11] Levet, P.N. (2001). *Leptospirosis*. Clinical Microbiology Review. Vol 14: 296-326.

Millar, B.D., Chappel, R.J. and Adler, B. (1987). Detection of leptospires in biological fluids using DNA hybridization. Veterinary Microbiology. Vol 15: 71-78.

[12] Merien, F., Amouriauz, P., Perolat, P., Barnaton, G. and Saint Girons, I. (1992). Polymerase chain reaction for detection *Leptospira* spps. in clinical samples. Journal of Clinical Microbiology. Vol 30:2219-2224.

[13] Noubade, R., Krishnamurthy, G.V., Murag, S., Venkatesha, M.D. and Krishnappa, G. (2002). Indian Journal of Medical Microbiology. Vol 20(1):33-36.

[14] Pacciarini, M.L., Savio, M.L., Tagliabue, S. and Rossi, C. (1992). Repetitive sequences cloned from *Leptospira interrogans* serovar *hardjo* genotype hardjoprajitno and their application to serovar identification. Journal of Clinical Microbiology. Vol 30: 1243–1249.

[15] Perolat, P., Grimont, F., Regnault, B., Grimont PADE, Fournie Thevenet, H. and Baranton, G. (1990). rRNA gene restriction patterns of *Leptospira*: a molecular typing system. Research Microbiology. Vol 141: 159–171.

[16] Shukla, J., Tuteja, U. and Batra, H.V. (2003). 16S rRNA PCR for differentiation of pathogenic and nonpathogenic leptospiral isolates. Indian journal of Medical Microbiology. Vol 21(1):25-30.

[17] Silva, M.V., Nakamura, P.M., Camargo, E.D., Balisk, L., Vaz, A.J., Romero, E.C. and Branolao, A.P. (1997). Immunodiagnosis of human leptospirosisi by dot-ELISA further detection of IgM, IgG and IgA antibodies. Amercian Journal of Tropical Medicine Hygeine. Vol 56: 650-655.

[18] Terpstra, W.J., Schoone, G.J., Ligthart, G.S. and Ter Schegget, J. (1987). Detection of *Leptospira interrogans* in clinical specimens by in situ hybridization using biotin-labelled DNA probes. Journal of General Microbiology. Vol 133: 911–914.

[19] Teresa Rocha Ermelinda., Cardeso Antonio M Terrinha Jose F Mouranunes., Kari Hovind., Hougen. and Marina Cinco. (1993). Isolation of new serovar of the genus leptonema in the family leptospiraceae. Journal of Zentravlakt Backterioloie. Vol 279: 167-172.

[20] Turk, N., Milas, Z., Margaletic, J., Staresina, V., Slavica, A., Riquelme Sertour, Bellenger, E., Baranton, G. and Postic, D. (2003). Molecular characterization of leptospira species strains isolated from small rodents in Croatia. Epidemiology and Infections, Cambridge Journal. Vol 130: 159-166.

[21] Turner, L.H. (1970). Leptospirosis III Maintenance, isolation and demonstration of leptospires. Trans Royal Society of Tropical Medicine and Hygiene. Vol 64: 623-646.

[22] Van Eys, G.J.J.M., Zaal, J., Schoone, G.J. and Terpstra, W.J. (1988). DNA hybridization with hardyobovis - specific recombinant probes as a method for type discrimination of Leptospira interrogans serovar hardgo. Journal of General Microbiology. Vol 134: 567-574.

[23] Van Eys, G.J.J.M., Gerristen, M.J., Korver, H., Schoone, G.J., Kroon, C.C.M. and Terpstra, W.J. (1991). Characterization of sreovars of the genus Leptospira by DNA hybridization with hardjobovis and icterohaemorrhagiae recombinant probes with special attention to serogroup serjroe. Journal of Clinical Microbiology. Vol 29: 1042-1048.

[24] Vinetz, J.M., Glass, G.E., Flexner, C.E., Mueller, P., Kaslow, D.C. (1996). Sporadic urban.

[25] Vinetz, J.M. (2001). Leptospirosis. current infectious disease. Vol 14: 527-538.

[26] Vijayachari, P., Sehgal, S.C., Goris, M.G., Terpstra, W.J. and Hartskeerl. (2003). Leptospira interorgans server valbuzzi : A cause of severe pulmonary haemorrhages in Andaman Islands Journal of Medical Microbiology. Vol 52: 913-918.

[27] Wangroongsarb, P., Supala Yaseang Wirrol Pefkanjanapong., Pimjai Naigowoit., Tshikatsu hagiwara., Hirokikawabbata. and Noboo Krizumi. (2005). Journal of Tropical Medicine Parasitology. Vol 28: 43-47.

[28] Zuerner, R.L. and Bolin ,C.A. (1988). Repetitive sequence element cloned from *Leptospira interrogans* serovar *hardjo* type hardjo-bovis provides a sensitive diagnostic probe for bovine leptospirosis. Journal of Clinical Microbiology. Vol 26: 2495–2500.

[29] Zuerner, R.L. and Bolin, C.A. (1990). Nucleic acid probe characterizes *Leptospira interrogans* serovars by restriction fragment length polymorphisms.Veterinary Microbiology. Vol 24: 355–366.

Table 1: Details of clinical samples collected and leptospiral isolates recovered

S.No	Source of isolation	No. of samples subjected for isolation	No. of samples found positive	Percent positivity
1	Rats	299	5	1.67
2	Sheep	42	5	11.91
3	Pigs	15	4	26.6
4	Humans	53	2	3.77
5	Rice field	10	1	10
6	Cattle	26	-	-
7	Dogs	13	-	-

Table 2: Results of 16S rRNA of Leptospiral isolates

Leptospiral isolate	Samples		Molecular diagnostic test
	Source	Type of material	16S rRNA
S1	Sheep	Blood	+
S2	Sheep	Blood	+
S3	Sheep	Blood	+
S4	Sheep	Blood	+
S5	Sheep	Blood	+
RR1	Rat	Kidney	+
RG1	Rat	Kidney	+
RR2	Rat	Kidney	+
RG2	Rat	Kidney	+
RG3	Rat	Kidney	+
P1	Pigs	Aborted material	+
P2	Pigs	Aborted material	+
P3	Pigs	Aborted material	+
P4	Pigs	Aborted material	+
H1	Humans	Blood	+
W1	Rice field	water sample	-
H2	Human Blood	Blood	*

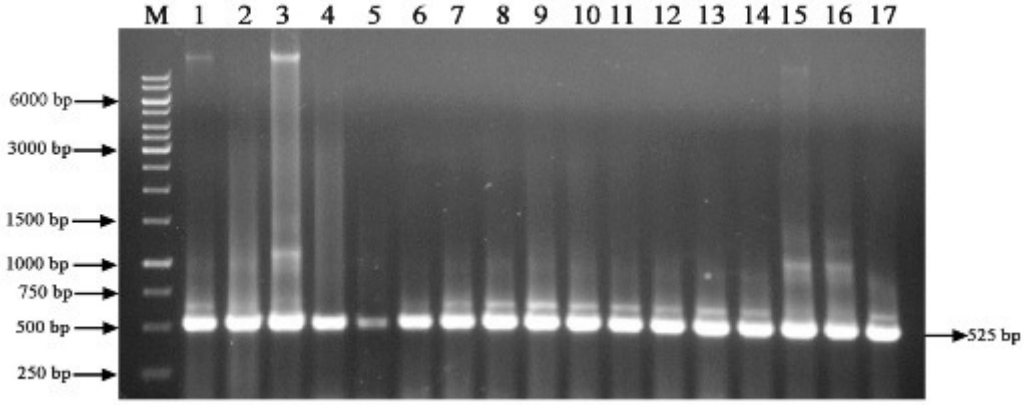
*** Not subjected to 16S rRNA**

Table 3: Sequence results of representatives of the leptospiral isolates

Isolate	RAPD	16S rRNA
S1 (1)*	*L.autumnalis*	*Leptonema illini*
S3 (3)*	*L.hardjo*	*L.hardjo*
S4 (6)*	*L.pomona*	*L.inadai*
RR1 (8)*	Separate banding pattern	*L.noguchi* strain panama
RG1 (10)*	Separate banding pattern	*L.illini*
P4 (16)*	*L.pomona*	*L.pomona*

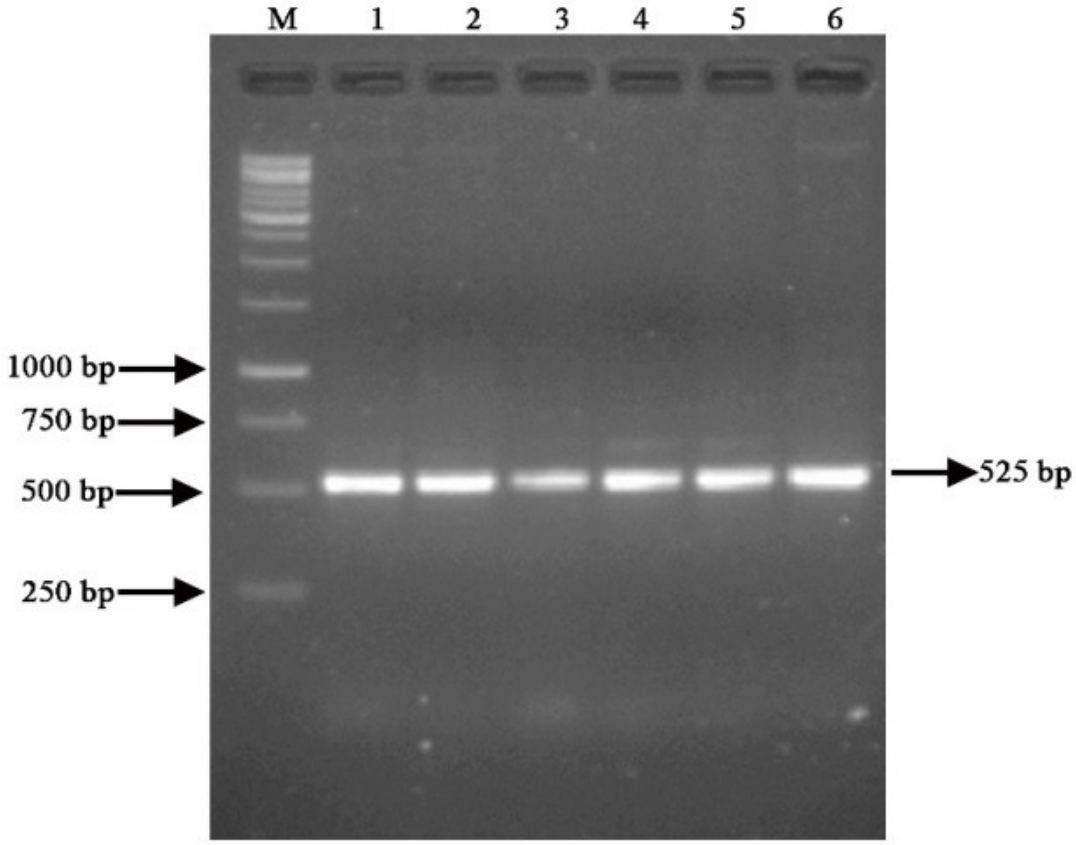
- Sample numbers sent for analysis

Fig.1: 16S rRNA – PCR for leptospiral isolates



M	**:**	**Molecular weight marker 250 bp**
1	**:**	**S1 isolate**
2	**:**	**S2 isolate**
3	**:**	**S3 isolate**
4	**:**	**RR2 isolate**
5	**:**	**RG2 isolate**
6	**:**	**S4 isolate**
7	**:**	**S5 isolate**
8	**:**	**RR1 isolate**
9	**:**	**RR3 isolate**
10	**:**	**RG1 isolate**
11	**:**	**H1 isolate**
12	**:**	**W1 isolate**
13	**:**	**P1 isolate**
14	**:**	**P2 isolate**
15	**:**	**P3 isolate**
16	**:**	**P4 isolate**

Fig.2: 16S rRNA – PCR for leptospiral isolates sent for sequencing



M : Molecular weight marker 250 bp

1 : Sample 1 (S1 isolate)

2 : Sample 3 (S3 isolate)

3 : Sample 6 (S4 isolate)

4 : Sample 8 (RR1 isolate)

5 : Sample 10 (RG1 isolate)

6 : Sample 16 (P4 isolate)