Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jls.htm
2015 Vol. 5 (3) July-September, pp.6-14/Rezasoltani et al.
Research Article

MOLECULAR TYPING OF *LEPTOSPIRA INTROGANS* ISOLATES BY MULTILOCUS VARIABLE –NUMBER TANDEM-REPEAT ANALYSIS (MLVA) IN IRAN

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ABSTRACT
Leptospirosis is the zoonotic disease which is characterized as a re-emerging infectious disease with high outbreaks. Today's MLVA method (multilocus variable number tandem repeat analysis) is used for identifying of *Leptospira* serovares. This simple PCR-based technique is a powerful methodology for the epidemiological investigation. The purpose of current study is molecular typing of selected isolates by MLVA for molecular epidemiology purpose. *L. interrogans* isolates were obtained from livestock from different regions of Iran during March 2013 to August 2014 and was stored at Razi institute. MAT **(**micro aglotination test**)** was performed. *Leptospira* were sub cultured into the liquid EMJH medium and incubated at 28^0C for 7 days. Genomic DNA of samples was extracted by phenol-chloroform method. PCR was performed with the 10 selected VNTR loci. The sizes of the amplified products were estimated by agarose gel electrophoresis and comparison with a 100-bp ladder and completed by sequencing analysis. Serovares of *Leptospria interrogans* were identified by MAT. All loci successfully amplified in all pathogenic *Leptospira* serovars. The saprophytic serovares showed no amplified fragments. The serovares were typed by the numbers of variable tandem repeats in each of the ten VNTR loci. The most diversity was observed in five loci out of ten. The study showed that there is high diversity of *Leptospira* serovars in Iran. Based on this study MLVA showed the high repetitive and discriminative power for *L. introgans*. All of studied isolates were totally clustered in different taxa, thereby it is concluded that VNTR method can provide rapid typing as well as a highly discriminant assay to identify *L. interrogans* serovars in large scales for epidemiological investigation.

Keywords: *MLVA Technique, Leptospira Serovares, VNTR Loci, Epidemiological Investigation*

INTRODUCTION
Leptospirosis is a planetary important public health menace, both in developed and under developing countries. It seems to be troublesome disease control because *Leptospires* will survive for long time in a wide range of environmental reservoirs, and both wild and domestic animals are important transfer agents of the disease ((Rafiei *et al.*,2012; Supply *et al.*, 1997). *Leptospires* are shed in to the environment via the urine of these animals, where they survive in the soil or fresh water. Human infections result from contact with the contaminated soil/water or from direct contact with animals or their infectious body fluids. The genus *Leptospira* consists of pathogenic and saprophytic species related to S*pirochaetales* order. DNA variations causes differentiation between species under high stringency conditions (Brenner *et al.*, 1999; Levett, 2001; Yasuda *et al.*,1987) Recently,12 named species of *Leptospires (L.biflexa, L.alexanderi, L. interrogans, L.inadai, L.fainei ، L. borgpetersenii, L. santarosai, L. noguchii, L. kirschneri, L.wolbachii, L. meyeri, L. weilii*) are classified according to genetic pattern, which *L. interrogans* includes the most pathogenic serovares and greatest dispersion in the world (Brenner *et al.*,1999). The prevalence of disease in Iran is in the northern provinces of Caspian Sea by having mild and wet weather conditions, this disease is more spread than other parts of the country. Today's with evolution of the *Leptospira,* disease from traditional village that enhance its prevalence among farmers and fishing, modified to epidemics in

urban communities that do not have good health. The most frequent *Leptospira* serovars in Mazandaran province includes Balum, Sejeroe, Tavasovi, Australis (Faraji, 2009). Serving leptospirosis in livestock around of Tehran in 1992 revealed dominant serovares of *Leptospira interrogans* is Hardjo, Tavasovi, Grippotyphosa, Pomona and Icterohaemorrhagia (Vandyousefi *et al.*, 1994). Identification of *Leptospires* has conventionally been performed by cultural and serological methods, microscopic agglutination test (MAT) which is gold standard for detection of *Leptospira (*Aghaiypour and Safavie, 2007*).* Rapid detection is a critical step for treatment and control of this disease. In this regards many PCR based method such as pulse filed gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), arbitrarily primed PCR (AP-PCR), florescent amplified fragment length polymorphism (FAFLP) and randomly amplified polymorphic DNA (RAPD) was evaluated for detection and identification of *Leptospira* serovars as well as molecular epidemiology (Herrmann *et al.*, 1991a, 1992b; Zuerner *et al.*, 1993). Recently multilocus variable number tandem repeat analysis (MLVA) provides useful tools in detection and identification of *Leptospira* serovars and gives information relating to both the evolutionary and functional areas of bacterial diversity (Leon *et al.*, 2010). Considering the high prevalence of leptospirosis in both human and livestock and lack of any study in case of molecular epidemiology of isolated strain here we aimed to molecular typing of selected isolates by MLVA technique for molecular epidemiology purpose.

MATERIALS AND METHODS

Samples and Isolates Culture

Sampling for Leptospira: L.interrogans isolates were obtained from cattle from different region of Iran during March 2013 to August 2014 and was stored at Razi institute. *Leptospira* serovars were cultured at 28°c in EMJH liquid medium (Difco, Germany) and were incubated up to 3 weeks to stationary phase (Herrmann, 1993) (table1).

MAT: MAT was performed according to standard methods using the main pathogenic serovares of *Leptospira* as live antigen. Then samples of study had been tested routinely with these live antigens at a standard density (Aghaiypour and Safavieh, 2007; Soltani *et al.*, 2012).

VNTR primer selection: In this study 10 loci of strongest loci which have been displayed the widest range of polymorphism among previous studies were selected for VNTR analysis which has been shown in table 2 (Salaum *et al.*, 2005 Majed *et al.*, 2005; Slack *et al.*, 2005a, 2006b; Elisa *et al.*, 2010).

Table 1: Serovars of *Leptospira interrogans* was identified in the current study

RTCC No	Species	Serogroup	Serovar	Strain	Abbreviation
2802	*L.inerrogans*	*Autumnalis*	Autumnalis	AkiyamiA	Aut
2805	*L.inerrogans*	*Canicola*	Canicola	Hondutrecht IV	Ch1
2808	*L.inerrogans*	*Grippotyphosa*	Grippotyphosa	Moskava	G1
2810	*L.introgans*	*Sejroe*	Hardjo	Hardjo bovis	Sh1
2812	*L.inerrogans*	*Icterohaemorrhagia*	Icterohaemorrhagia	Verdum	Ict1
2815	*L.inerrogans*	*Pomona*	Pomona	Pomona	Po1
2817	*L.inerrogans*	*Sejroe*	Serjae serjae	M84	Ser
2819	*L.biflexa*	*Semaranga*	Patoc	Patoc1	Pat
2821	*L.interrogans*	*Sejroe*	Hardjo	Hardjo prajitno	Sh2
2822	*L.inerrogans*	*Pomona*	Pomona	UT364	Po2
2823	*L.inerrogans*	*Icterohaemorrhagia*	Icterohaemorrhagia	RGA	Ict2
2824	*L.inerrogans*	*Canicola*	Canicola	Fiocruz LV133	Ch2
2825	*L.inerrogans*	*Grippotyphosa*	Grippotyphosa	Andaman	G2
2826	*Leptospira meyeri*	*Sejroe*	Hardjo	Went 5	H
2827	*Leptospira meyeri*	*Semaranga*	Semaranga	Veldrot Semaranga 173	V
2828	*Leptospira wolbachii*	*Codice*	Codice	CDC	C

***Razi Type Culture Collection**

Table 2: PCR primers used in this study

VNTR Locus	Sequence 5′→3′	Reference
VNTR4	4a(AAGTAAAAGCGCTCCCAAGA) 4b(ATAAAGGAAGCTCGGCGTTT)	(Salaum *et al.*, 2005)
VNTR LB5	Lb5a(AGCGAGTTCGCCTACTTGC) Lb5b(ATAAGACGATCAAGGAAACG)	(Salaum *et al.*, 2005)
VNTR 7	7a(TCATCTGCTCCGGAGATTCG) 7b(TCCCTCCACAGGTTGTCTTG)	(Majed *et al.*, 2005)
VNTR 8	F(CAAGTGTTCGACAAGAATGAG) R(CTCACCGGTAGAACGCTTCTTTT)	(Slack *et al.*, 2005)
VNTR10	10a(TCCAAAATTCAGCCCTCAAG) 10b(GACGCTTGGCATTTGTATCC)	(Majed *et al.*, 2005)
VNTR 19	19a(CAGAAACAAGAGGGAAGATTC) 19b(ACTCTCATTTAAGAGTGGCTG	(Majed *et al.*, 2005)
VNTR 29	F(ATCGTTTTGGCAGTTTTTGCT) R(CTAGAAAATTCCGCGTAGGG)	(Slack *et al.*, 2005)
VNTR30	F(AAGTAAGATAGGTTCGGCGTTTA) R(ACTTGGGTGTTAATCGCAAAA)	(Slack *et al.*, 2005)
VNTR36	(TGGCGTCGAAGACAAA) (ACTCTACCAGGAGATTATCAAA)	(Slack *et al.*, 2006)
VNTR50	F(CTTGTTGGATCACAATACGAACTATA) R(GGTAAGGGACAAAGTGAAGC)	(Slack *et al.*, 2006)

DNA Extraction

The Genomic DNA of *Leptospira* was extracted by common phenol-chloroform method (19), briefly organisms were centrifuged at 13,000 g for 30 min at 4 °C. The pellet was washed twice in phosphate-buffered saline (PBS) and DNA released from *Leptospires* with incubation of the bacteria in 200 µl of buffer.The released DNA was extracted with equal volume of phenol/choloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol 70% v/v. After complete removal of ethanol, the precipitated DNA was resuspended in 100 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA) pH 8.0 (Slack *et al.*, 2005).

PCR: For detection of VNTR, PCR was performed at the final volume of 25 µl containing 2/5 µl PCR buffer (Sina clone),0.5 mM deoxinucleoside triphosphates (sina clone),1µM each corresponding primer, 0/75MgCl2 (Sina clone), 0.3 unit/ µl of Taq DNA polymerase (Vivantis) and 1 µl of DNA template. Amplification was achieved under the following conditions: one denaturation cycle at 93°c for 5 min;35 cycles of denaturation at 93°c for 1 min, annealing at 54°c for 1 min, and elongation at 72°c for 1 min, and a final elongation at 72°c for 10 min. The amplified products were analyzed by 2% agarose gel electrophoresis and allelic sizes estimated by Quantity One 1D Analysis software package (Bio Rad). The sizes of the amplified products were estimated by comparison with a 100bp plus ladder (Majed *et al.*, 2005; Slack *et al.*, 2005).

Sequence Analysis

Some fragments of pathogenic serovares were sequenced at the macro gene company in South Korea by singer method. The copy number of repeats of each VNTR locus was deduced from sequencing data and sizes of the amplified products (Garcia *et al.*, 1999). The Clustal X program was used to generate nucleotide sequence alignments. The allelic sizes were then converted in to repeat copy numbers using Microsoft Excel software package. Using the formula: Number of Repeats(pb)=[Fragment size(pb)-Fanking regions(pb)]/Repeat size(pb). The repeat copy numbers were then rounded down to form whole numbers (table3). Clustering analysis was done using the categorical parameter and the ward coefficient. Nei,s Diversity Index of the VNTR loci was calculated from the range of alleles generated from the

reference strains utilizing the formula; D=1-[allele frequency) (table3)(20). Dendrogram of studied isolated was conducted based on VNTR results using Dendro UPGMA software (Slack *et al.*, 2005).

Table 3: Copy numbers of of VNTRs in all studied serovares

Serovares	VNTR4	VNTRLB5	VNTR7	VNTR8	VNTR10	VNTR19	VNTR29	VNTR30	VNTR36	VNTR50
***L. introgans* serovar Autumnalis (2802)**	4	6	12	4	11	2	6	5	9	3
***L. introgans* serovar Canicola (2805)**	2	4	11	1	5	9	5	2	13	5
***L. introgans* serovar Canicola (2824)**	2	4	12	1	5	8	5	3	10	6
***L. introgans* serovar Grippotyphosa (2808)**	3	6	3	1	4	9	3	2	9	5
***L. introgans* serovar Grippotyphosa (2825)**	1	7	12	1	5	7	5	3	10	5
***L. introgans* serovar hardjo (2810)**	3	6	12	1	13	14	10	5	0	6
***L. introgans* serovar Serjoehardjo (2821)**	2	6	12	1	4	9	5	3	13	5
***L. introgans* serovar Icterohaemorrhagiae (2812)**	2	6	12	1	5	9	5	3	13	5
***L. introgans* serovar Icterohaemorrhagiae (2823)**	4	7	5	1	9	1	6	4	10	6
***L. introgans* serovar Pomona (2815)**	2	3	13	1	5	9	5	3	13	6
***L. introgans* serovar Pomona (2822)**	5	7	5	1	11	7	3	1	0	4
***L. introgans* serovar Serjaeserjae (2817)**	2	7	13	5	5	9	7	3	13	6

RESULTS AND DISCUSSION

Results

Out of selected samples 16 isolates were positive for *Leptospira* spp. in culture. According to MAT results, 12 pathogenic and 4 saprophytes from 10 different serovares were identified which are listed in table1. Respect to each locus, analysis of the amplified PCR products revealed size variations among different isolates by agarose gel electrophoresis.

All of the loci exhibited a single PCR product in all of studied isolates. All loci successfully amplified in all pathogenic *Leptospira* serovars while saprophytic serovares including Hardjo, Semaranga, Codice and Patoc showed no amplified fragments. The sizes of the amplified products displayed range of polymorphism, suggesting variation in tandem repeat copy numbers in the ten VNTR loci. This was confirmed by sequencing of some amplified products from the ten loci. For each VNTR locus, Sequence analysis of amplified products displayed a high conservation of flanking regions and repeat units among *L.interrogans serovares*. For each locus, the number of tandem repeats was calculated by measuring the sizes of the amplified product which was displayed in (table 3).

The ten markers were able to differentiate 12of 16 (75%) *Leptospira interrogans* serovares.

The serovares were typed by the numbers of variable tandem repeats in each of the ten VNTR loci. Cluster analysis of the VNTR data showed that the studied *Leptospira* isolates samples comprise populations consisting of three main clusters grouped into related branches (Figure 1). Based on our results, although many of isolates (7 out of 12) were grouped in one branch, however, the same serovares were not clustered in same groups (figure 1).

The most diversity was observed in VNTR 19 followed by VNTR 7, VNTR30, VNTR29 and VNTR10 and the lowest diversity in VNTR8.

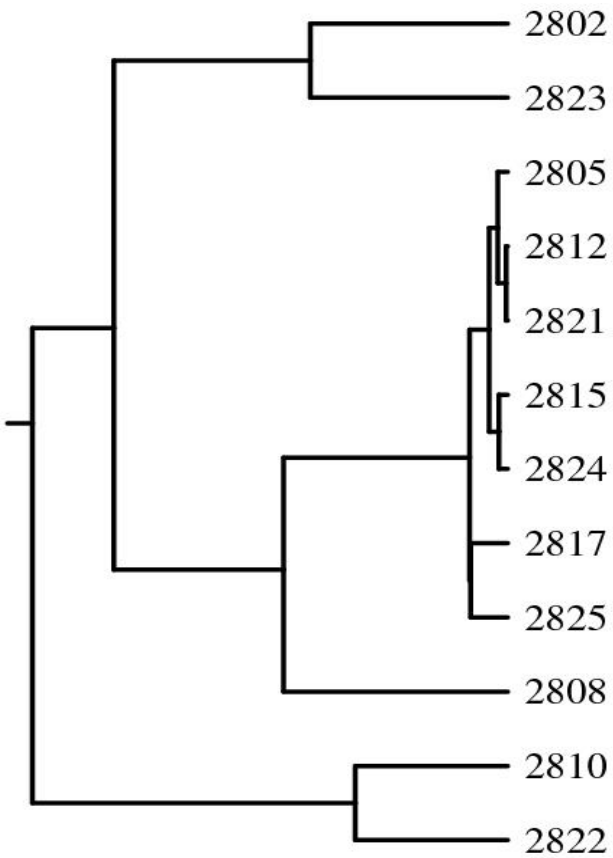

Figure 1: Cluster analysis of the studied *Leptospira* spp. Isolated based on VNTR results using UPGMA software

Discussion

Leptospirosis is an emerging infectious disease and is considered to be the most important zoonotic disease in the world. Epidemiological investigation is urgent for recognition outbreak situations or to trace reservoirs of the *Leptospira* (Brenner *et al.,* 1999; Levett, 2001). Recently MLVA method is used for differentiation and typing of *Leptospira* serovares. The method seems to have potential application for understanding molecular epidemiology of *Leptospira* with easy, rapid and highly discriminatory differentiation power (Majed *et al.,* 2005; Slack *et al.,* 2005). *Leptospira* genomes contain multiple loci of repetitive DNA which may be variable among various serovars with respect to their individual primary structure or numbers of repeat units present (Leon *et al.,* 2010).

The Indian subcontinent, southeast Asia, the Caribbean and Latin America, Oceania, and to a lesser extent Eastern Europe, appear to have the highest incidence of leptospirosis.The wet and warm climate in the south east Asia and also north of Iran are a favorable condition for *Leptospires* (Pappas *et al.,* 2008). Detection of 10 diverse serovares; 5 newly reported and 5 in share with previous report (Vandyousefi *et al.,* 1994). Among sixteen isolates of *Leptospira* in the current study suggesting the high diversity of *Leptospira* spp. in Iran.

In table 4 the most isolated serovars of leptospirosis in some endemic countries of the world were shown. Considering this table it is clear that distribution of *Leptospira* serovars due to wide range of reservoirs is not region restricted, for example we share same serovars with other countries such as Turkey, India and Argentina (KocabiIyik and Cetin, 2004; Petrakovsky *et al.,* 2014).

Table 4: Epidemiology of leptospirosis in some endemic countries of the world

Country	Reported serovars	Principal reservoirs	Reference
Iran			Current study
Iran	Grippotyphosa, Canicola, Hardjo, Icterohaemorrhagia	livestock	(Vand *et al.,*1994)
Turkey	hardjo, grippotyphosa, Pomona	cattle, Bovine	(KocabiIyik, 2004)
India	icterohaemorrhagiae, Copenhageni autumnalis, canicola, pyrogenes, grippotyphosa, australis, javanica, sejroe, louisiana, pomona, valbuzz	Rodents, pigs, cattle, bandicoots, dogs	World health organization (2008)
Malaysia	Icterohaemorrhagiae, mankarsoa, smithii and birkin	livestock	(1El Jalii, 2004)
Thailand	icterohaemorrhagiae, grippotyphosa, autumnalis, hebdomatis, ranarum, pyrogenes, australis, javanica, sejroe, bratislava, Pomona	Rodents, bandicoots, cattle, buffalo, pigs	(World health organization, 2008)
Sri Lanka	icterohaemorrhagiae, grippotyphosa, autumnalis, canicola,	Rodents, cattle, buffalo, dogs	(World health organization 2008)

	australis, javanica, sejroe, Louisiana, pyrogenes, hardjo, hebdomatis		
Argentina	Icterohaemorrhagiae, Canicola, Pomona,	Squirrels, Cows, Pigs, South American gray fox (Lycalopex griseus), Dog fetus	(Petrakovsky *et al.*, 2014)
Brazil	*L.borgpetersenii* serovar Castellonis, *L.interrogans* serogroup Icterohaemorrhagiae, *L. interrogans* serovar Canicola and Copenhageni, L. kirshneri serovar Grippotyhosa, *L. interrogans* serovar Canicola, *L.noguchi* serogroup Autumnalis, *L. noguchi*, *L. interrogans* serovar Canicola	Marsupials, Capybara (Hydrochoerus hydrochaeris), Cattle, Swine, Sheep, Dogs	(Petrakovsky *et al.*, 2014)
Mexico	L. kirshneri serovar Hardjo	Cattle	(Petrakovsky *et al.*, 2014)
Peru	*L. licerasiae* serovar Varillal, *L. interrogans, L. kirshneri, L. borgpetersenii and L. fainei*	Rattus norvegicus, Rattus rattus, Bats	(Petrakovsky *et al.*, 2014)
Trinidad and Tobago	*L. interrogans* serovar Copenhageni	Rattus norvegicus, Rattus rattus,	(Petrakovsky *et al.*, 2014)

Our saprophytic serovars showed no amplified fragments and this is similar to what was obtained by Majed, Slack and Salaun for their saprophytic serovares (Salaum *et al.*, 2005; Majed *et al.*, 2005; Slack *et al.*, 2005a, 2006b). Thereby the lack of VNTRs in saprophytic serovares seems to be good marker for differentiation between pathogenic and non-pathogenic serovares. More over since only pathogenic isolates were positive for these loci, hence this region may have a role in pathogenesis of this bacterium.
Considering the discriminatory power of each VNTRs, our study showed that VNTR19 followed by VNTR 7, VNTR30, VNTR29 and VNTR10 with highest range of different allelic size offer better typing power for typing of *L.introgans* among other studied VNTR. This finding is in agreement with Majed *et al.*, in which VNTR7, VNTR10 and VNTR19 was proposed with the highest discriminatory power.
When we analyzed VNTRs pattern for some selected *Leptospira* isolates from different countries in integration with our result by UPGMA software, interestingly the dendrogram results showed the same serovares from different countries are clustered in same places. This is proven for repetitively power of MLVA typing for *Leptospira* (Salaum *et al.*, 2005; Majed *et al.*, 2005). Also in the current study we analyzed the same sevares considering VNTRs by MLVA, and found that in some cases they were clustered in distinct taxa. Since there is no other study in which same serovars has been admitted to MLVA, so we conclude that this may contribute the diversity in Iranian serovars or even MLVA limitation. However for precise conclusion the larger number of serovars is needed.
As all of studied isolates were totally clustered in different taxa, thereby we can conclude that VNTR method can provide rapid typing as well as a highly discriminant assay to identify *L. interrogans* serovars

in large scales for epidemiological investigation. This finding is in agreement with majority of studies in which the VNTR has been introduced as a powerful method for molecular typing of *Leptospira* isolates (Salaum *et al.,* 2005; Majed *et al.,* 2005; Slack *et al.,* 2005a, 2006b; ElisaPavan, 2010).

Conclusion

Our finding is in contrast with those studies in which they have been shown that serovares from the same geographical area could have more genetic similarity than the serovares in different places such as South America and Europe. On the other hand our data did not support the idea that strains originating from distant continent can be grouped together because of same area (Salaum *et al.,* 2005; Majed *et al.,* 2005; Slack *et al.,* 2005). This study showed there is high diversity of *Leptospira* serovars in Iran. Of all studied VNTRs, the VNTR 19 followed by VNTR 7, VNTR30, VNTR29 and VNTR10 with highest range of different allelic size offered better differentiation power within serovares and strains. Based on our study MLVA showed the high repetitive and discriminative power for *L.introgans* and thereby we recommend the MLVA as appropriate tools for molecular typing of *L. introgans* serovaes.

ACKNOWLEDGEMENT

The authors would like to thank Department of Microbiology, Faculty of Basic Sciences, Zanjan Branch, Islamic Azad University, Zanjan, Iran

Competing interests: The authors have no conflict of interest to disclose.

REFERENCE

Aghaiypour K and Safavie S (2007). Molecular detection of pathogenic *Leptospira* in Iran. *Archives of Razi Institute* **62**(4) 191-197.

Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC and Weyant RS (1999). Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. *International Journal of Systematic Bacteriology* **49**(3) 839–858.

ElisaPavan M, FabiánCairó M, Pettinari M, Samartino L and Brihuega B (2011). Genotyping of *Leptospira interrogans* strains from Argentinaby Multiple-Locus Variable- number tandem repeat Analysis (MLVA). *Comparative Immunology, Microbiology and Infectious Diseases* **34**(2) 135–141.

Faraji H (2009). Prevalence of leptospirosis in Mazandaran province, using micro agglutination and indirect fluorescence immunoassay. *Iranian Journal of Biology* **22**(4) 608-618.

Garcia S, Vallve J, Palau A and Romeu (1999). Horizontal gene transfer in glycosyl hydrolases Inferred from codon usage in Escherichia coli and Bacillus subtilis. *Molecular Biology and Evolution* **17**(3) 352-61.

Herrmann JL (1993). Genomic techniques for identification of *Leptospira* Strains. *Pathologie Biologie* **41**(10) 943–950.

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

Jalii IM and Bahaman AR (2004). A Review of Human Leptospirosis in Malaysia. *Tropical Biomedicine* **21**(2) 113-9.

KocabiIyik AL and Cetin C (2004). Bovine leptospirosis in south Marmara region of Turkey: A serological survey. *Revuede Medecine Veterinaire* **155**(12) 606-608.

Leon A, Pronost S, Guillaume F, Genevie`ve Andre F and Leclercq R (2010). Multilocus Sequence Analysis for Typing *Leptospira interrogans* and *Leptospira kirschneri. Journal of Clinical Microbiology* **48**(2) 581–585.

Levett PN (2001). Leptospirosis. *Journal of Clinical Microbiology* **14**(2) 296–326.

Majed Z, Bellenger E, Postic D, Pourcel C, Baranton G, Picardeau M (2005). Identification of variable-numbertandem-repeatlociin *Leptospira interrogans sensu strict*. *Journal of Clinical Microbiology* **43**(2) 539–545.

Pappas G, Papadimitriou P, Siozopoulou V, Christou L and Akritidis N (2008). The globalization of leptospirosis: worldwideincidence trends. *International Journal of Infectious Dis*ease **12**(4) 351—357.
Perolat P, Lecuyer I, Postic D and Baranton G (1993). Diversity of ribosomal DNA fingerprints of *Leptospira* serovars provides a database for subtyping and species assignation. *Research in Microbiology* **144**(1) 5–15.
Petrakovsky J, Bianchi A, Fisun H, Nájera-Aguilar P and Pereira M (2014). Animal Leptospirosis in Latin America and the Caribbean Countries: Reported Outbreaks and Literature Review. *International Journal of Environmental Research and Public Health* **11**(10) 10770-89.
Rafiei A, Hedayati Zadeh-Omran A, Babamahmoodi F, Alizadeh Navaei R and Valadan R (2012). A Review of Leptospirosis in Iran. *Journal of Mazandaran University of Medical Science* **22**(94) 113-124.
Ralph D, McClelland M, Welsh J, Baranton G and Perolat P (1993). *Leptospira* species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. *Journal of Bacteriology* **175**(4) 973–981.
Salaum L, Merien F, Gurianova S and Picardeau BG (2010). Application of multilocus variable number-tandem repeat analysis for molecular typing of the agent of leptospirosis. *Journal of Clinical Microbiology* **44**(11) 3954-62.
Slack A, Dohnt S and Smythe (2005). Development of a Multiple-locus Variable number of tandem repeat Analysis(MLVA) for *Leptospira interrogans* and its application to *Leptospira interrogans* serovar Australis isolates from Far North Queensland, Australia. *Annals of Clinical Microbiology and Antimicrobials* **4** 10.
Slack A, Symonds M, Dohnt M and Smythe L (2006). An improved multiple-locus variable number of tandem repeats analysis for *Leptospira interrogans* serovar Australis: a comparison with fluorescent amplified fragment length polymorphism analysis and its use to redefine the molecular epidemiology of this serovar in Queensland, Australia. *Journal of Medical Microbiology* **55**(11) 1549–1557.
Soltani Majd N, Khodaverdi Darian E and Khaki P (2012). Epidemiological patterns of Leptospira spp. among slaughterhouse workers in Zanjan- Iran. *Asian Pacific Journal of Tropical Disease* **2**(2) 550-552.
Supply P, Magdalena J, Himpens S and Locht C (1997). Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. *Molecular Microbiol*ogy **26**(5) 991–1003.
Thiermann AB, Handsaker AL, Moseley SL and Kingscote B (1985). New method for classification of *Leptospiral* isolates belonging to serogroup pomona by restriction endonuclease analysis: serovar kennewicki. *Journal of Clinical Microbiology* **21**(4) 585–587.
Vandyousefi J, Moradi Bidhendi S and Ahourai P (1994). New findings about Leptospirosis in Razi institute, *Pajouhesh and Sazandegi* **2**(1) 72-75.
World Health Organization (2008). Leptospirosis situation in the WHO south east Asia region. SEA-CD-216
Yasuda PH, Steigerwalt AG, Sulzer KR, Kaufmann AF, Rogers FC and Brenner DJ (1987). Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. *International Journal of Systematic and Evolutionary Microbiology* **37**(4) 407–415.
Zuerner RL, Herrmann JL and Saint Girons I (1993). Comparison of genetic maps for two *Leptospira interrogans* serovars provides evidence for two chromosomes and intraspecies heterogeneity. *Journal of Bacteriology* **175**(17) 5445–5451.