TABLE 1. Diarrheal clusters due to Sapovirus in 2011

No.	Sex	Age	Onset Date	Cluster City	Cluster institution	Sapovirus Genotype
SV-18-YL	F	32	Feb 24	Yilan County	Hiten Hospital	GI/2
SV-21-YL	M	30	Feb 25			
SV-22-YL	F	55	Feb 25			
SV-23-YL	F	71	Feb 25			
SV-24-NTP	F	53	Feb 11	New Taipei City	Bali Psychiatric center	GI/2
SV-25-NTP	F	55	Feb 6			
SV-26-NTP	F	70	Feb 16			
SV-27-NTP	F	47	Feb 18			
SV-28-NTP	F	57	Feb 13			
SV-72-CY	M	7	Feb 17	Chiayi County	Dalin elementary school	GI/3
SV-75-CY	F	6	Feb 17			
SV-77-CY	F	7	Feb 16			
SV-78-CY	M	7	Feb 17			
SV-82-YL	F	6	Mar 4	Yilan County	Gu Ting Kindergarten	GI/2
SV-84-YL	M	5	Mar 6			
SV-109-TP	F	6	Apr 22	Taipei City	Green-hill Nursery	GI/2
SV-151-PT	M	11	May 8	Pingtung County	Poachien Hospital	GV
SV-179-NT	M	20	Jun 23	Nantou County	Puli Hospital	GI/3

Development of diagnostic methods for brucellosis and leptospirosis

Chia-Ling Chen[1], Shih-Hui Chiu[1], Koichi Imaoka[2], Koizumi Nobuo[3]and Jung-Jung Mu[1*]

[1] Bacterial Enteric and Emerging Diseases Laboratory, Research and Diagnostic Center, Centers for Disease Control, Taiwan

[2] Department of Veterinary Science, National Institute of Infectious Disease

[3] Department of Bacteriology I, National Institute of Infectious Disease

Email:

Chia-Ling Chen:clchen@cdc.gov.tw

Shih-Hui Chiu:shihhui@cdc.gov.tw

Koichi Imaoka: imaoka@nih.go.jp

Koizumi Nobuo: nkoizumi@nih.go.jp

Jung-Jung Mu:jjmu@cdc.gov.tw

*Correspondence: Jung-Jung Mu

Mailing address: 161 Kun-Yang Street, Taipei, Taiwan

Tel:886-2-26531356 FAX:886-2-27864367

Keywords: brucellosis, leptospirosis

Abstract

Leptospirosis and brucellosis are important zoonotic infections. Lack of sensitive and rapid diagnostic methods, these two diseases are largely underdiagnosed and underreported. To improve the case detection, we need sensitive diagnostic methods and better understand the epidemiology of these diseases in Taiwan.

Introduction

Leptospirosis is an important global zoonosis caused by pathogenic *Leptospira* species. The genus *Leptospira* contains pathogenic and non-pathogenic (including saprophytic and intermediate) species that differ in their capacity for survival in various environments that ranging from soil and water to the tissues of mammalian hosts during acute and chronic infection (13). Pathogenic *Leptospira* can colonize in the proximal renal tubules of mammalian hosts and be excreted in the urine to the environment. Human infection can be acquired via direct contact with an infected animal or more frequently via indirect contact with environmental soil and surface waters contaminated by urine of infected mammalian hosts (4).

Conventional laboratory diagnosis for leptospirosis, which is time-consuming and laborious, usually depends on culture and serological techniques like microscopic agglutination test (MAT). For the culture technique, it can take up to two months and is very laborious with a low isolation rate (approximately 3%) (6). The main serological method, MAT, does not contribute to early diagnosis of leptospirosis, as antibodies become detectable on approximately the seventh day after infection (1) (9). Moreover, patients with fulminant leptospirosis may die before seroconversion occurs (4). Consequently, a rapid and accurate method for early diagnosis is urgently required to improve the timely problem.

Brucellosis is the most common zoonotic disease worldwide with more than 500,000 new cases diagnosed annually. This disease is caused by *Brucella* spp. and major animal hosts are goats, sheep, camels, cows and pigs. Transmission to humans occurs through ingestion of infected, unpasteurized animal-milk products, through direct contact with

infected animals or inhalation of infected aerosolized particles (10).

Surveillance and control of animal brucellosis in Taiwan was started in 1962. The first case of bovine brucellosis caused by *B.abortus* was diagnosed in 1963. After surveillance for more than one decade, the infection rate of bovine brucellosis has decreased from 4.99% in 1962 to 0.06% in 1979. Bovine brucellosis was eventually eradicated in Taiwan in 1989. Serological survey on sheep has been regularly performed since 1986 and all showed negative results. First domestic case of human brucellosis was reported in 1978 due to laboratory accident. First and the only outbreak occurred in 1979, in which 16 people including 9 laboratory personnel, 6 dairy farm workers and 1 veterinarian were infected. Since then, no human case, either domestic or imported, has ever been reported in Taiwan until May to Sep 2011, first four cases of human brucellosis in 30 years have been confirmed in Taiwan. All cases occurred in returned travelers from regions where brucellosis is endemic. It reminds us of the possible threat of this neglected zoonosis.

In this report, we develop a rapid diagnostic method for early diagnosis of leptospirosis, the LipL32 real-time PCR, by using TaqMan chemistry to detect *Leptospira* LipL32 gene in clinical samples. For diagnosis of Brucellosis, serum agglutination test and ELISA were used to detect Brucella infection in reported cases and exposed laboratory staff. These techniques improved diagnosis of leptospira and brucella infection in Taiwan and could help to establish surveillance system of leptospirosis and brucellosis in the future.

Methods

Leptospira strains and culture conditions

Eighteen Leptospira serovars from five pathogenic species (L. borgepetersenii, L. interrogans, L. santarosai, L.kirschneri and L. noguchii), and two non-pathogenic species (one is L. inadai which belonging to the intermediate species and the other is L. biflexa which belonging to a saprophytic species) from Centers for Disease Control in Taiwan were used in this study (Table 1). All cultures were grown in Ellinghausen-McCullough-Johnson-Harris broth (EMJH) and maintained by weekly subculture into fresh medium. Cultures used were grown up to seven days at 28°C to a density of approximately 1 x 10^8-5 x 10^8 leptospires/mL.

Non-leptospiral bacterial species

Twenty-two bacterial strains other than members of the genus Leptospira from Centers for Disease Control in Taiwan or American Type Culture Collection (ATCC) (Table 2) were used for specificity test.

DNA extraction from bacterial strains

Genomic DNA from one ml of culture medium of each leptospiral strain was centrifuged at 12,000g for 15 min at 4 °C or from the pellet of non-leptospiral bacterial strains was extracted by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacture's instructions.

DNA extraction from human plasma samples

Total DNA of human plasma (200 uL) was extractedd using QIAamp DNA Mini Kit according to the manufacture's instructions. DNA was eluted in a final volume of 200 uL.

Reconstitution experiments with human plasma

Pooled human plasma from normal healthy people was seeded with pathogenic Leptospira (Leptospira interrogans serovar Icterohaemorrhagiae strains CF-1), counted by a Petroff-Hauser (Hauser Scientific, USA) counting chamber, and serially diluted with pooled plasma to make 1 x 10^8 to 1 leptospires/mL of plasma. DNAs from all reconstituted samples were extracted as described for the real-time PCR.

Design of real-time PCR primers and probe

LipL32 sequences from the following serovars (Table 3) were aligned by using ClustalW. The TaqMan probe and primers were both designed and synthesizes by TIB Molbiol (TIB MOLBIOL Syntheseslabor, Berlin, Germany): LipL32-F (5'-GAAATGGGAGTTCGTATGATTTCC-3'), LipL32-R (5'-GGTTTTGCTTTCGCAGCTT-3') and TaqMan probe (5'FAM-TAATCGCCGACATTCTTTCTACACGGATC-3'BHQ). PCR will result in a 194 bp amplicon between position 274 and 467 of the LipL32 coding region.

Real-time PCR assay

Real-time PCR assay was performed using the Roche LightCycler version 2.0. The PCR mixture was prepared using a ready-made mastermix, to give a final concentration of 1 X LightCycler FastStart DNA Master HybProbe Mix (Roche Diagnostics, Mamnheim, Germany), 3mM MgCl2, 500 nM of each primer and 200 nM of the TaqMan probe. 10 uL of PCR master mix and 10 uL of DNA extract were loaded into a LightCycler capillary and centrifuged to mix. One ng DNA from Leptospira interrogans serovar Icterohaemorrhagiae strains CF-1 as positive control and 10 uL of ddH2O as no DNA control were used in each batch of samples. PCR amplification was performed using the parameters as follows. An initial denaturation/hot-start Taq activation at 95 °C for 8 min,

followed by 50 cycles of denaturation at 95 °C for 8 s and annealing/extension at 60 °C for 40 s, and a final cooling step at 40 °C for 30 s.

Positive samples were defined as having a cycle-to-threshold (CT) value between 5 and 40 cycles. Negative samples were defined as having a CT value greater than 50 cycles. Equivocal samples were defined as having a CT value between 40 and 50 cycles. All positive and equivocal samples were rerun with the re-extracted DNA from the original plasma sample.

Leptospira culture and microscopic agglutination test

Leptospira culture was performed using semisolid EMJH media plus 200 μg/ml 5-fluorouracil and cultured at 28 °C. At weekly intervals for up to 12 weeks, the cultures were analyzed for evidence of leptospires' growth, which indicated by the presence of typical morphology of spirochaetes under dark-field microscopy. All serum samples were subjected to a microscopic agglutination test (MAT) and had a starting dilution of 1:100 and a diagnostic result declared where there was a fourfold change between acute and convalescent sera. Twelve selected reference Leptospira serovars (serogroup, serovar, strain name) listed as follows: Canicola, Canicola, Hond Utrecht IV; Australis, Bratislava, Jez Bratislava; Pomona, Kennewicki, LT1026; Icterohaemorrhagiae, Icterohaemorrhagiae, CF-1; Semaranga, Patoc, Patoc 1; Shermani, Shermani, CCF; Lyme, Lyme, ATCC 43289; Javanica, Poi, Poi; Tarassovi, Tarassovi, Perepelistin; Pomona, Pomona, Pomona;Bataviae, Bataviae, Van Tienen; and a local strain 2939 which serovar has not been determined yet.

Microplate Agglutination Test for Brucellosis

Two fold serially diluted patients' sera were incubated with heat-inactivated B. abortus

strain 99 or 125 whole cells in a U-bottom 96 well culture plate. The sealed plate were mixed and incubated at 37℃ for 18~24 hr in a humid atmosphere. An agglutination titer greater than 40 was considered positive.

Results

(1) Leptospirosis

Specificity of the real-time PCR assay. To test the ability of the TaqMan probe and primers set in detection of lipL 32 target gene pathogenic Leptospira, total genomic DNA from 18 reference leptospiral strains belonging to five pathogenic species and two non- pathogenic species, listed in Table 1, were extracted and assayed. The data showed that only pathogenic leptospira could be detected by this LipL32 The specificity of the assay was also evaluated with DNA extracts from 22 different bacterial strains and no amplification was shown (Fig. 1). All products of positive samples, visualized by gel electrophoresis (5 uL of each PCR product on a 1.5% agarose gel containing ethidium bromide), showed a unique 194-bp band (data not shown).

Sensitivity of the real-time PCR assay To test the sensitivity of this real-time PCR, dilutions of a leptospira bacterical culture (Leptospira interrogans serovar Icterohaemorrhagiae strains CF-1) in pooled plasma from healthy people were used to mimic clinical samples. The standard curve for quantification was ranging from1 x 10^8 to 1000 leptospires/mL of plasma, and the limit of sensitivity was found to be 1000 leptospires /mL of plasma (Fig. 2) which concentration was approximately equal to 10 leptospires per reaction.

A standard curve of the CT values was obtained from the reconstitution experiments (1 x 10^8 to 1 leptospires/mL). Standards were run in triplicate to generate a standard curve. The CT values from unknown samples were plotted on the standard curves, and the number of leptospires per milliliter was calculated.

(2) Brucellosis

Between May and Sep. 2011, four cases of human brucellosis were reported to Taiwan CDC.(3) The first two Brucellosis cases were sent to Bureau of Animal and Plant Health and Quarantine, Taiwan for confirmation. In order to strengthen the ability for Brucellosis diagnosis, brucellosis antibody diagnostic kits were kindly provided by Dr. Koichi Imaoka from NIID, Japan. Microplate agglutination test was performed to detect antibody against Burcella infection in four reported cases and 21 exposed laboratory personnel. The four reported cases were all positive with titer 1:160, 1:80,1:160 and 1:80. Three laboratories encounter positive isolates of brucella (first three cases), and sera from those laboratory staff were sent to Taiwan CDC for health surveillance. After serological follow-up, no laboratory infection was found.

Discussion

The main purpose of this study was to develop a rapid diagnostic method of human leptospirosis in early phase. Considering the advantage of PCR technology, we choose a TaqMan probe based real-time PCR targeted to LipL32 as our first choice. The specificity and sensitivity of our real-time PCR assay was similar to other experiments' data (5, 7, 11, 12). To detect pathogenic leptospires by real-time PCR offers considerable benefits compared to conventional methods in diagnostic laboratories. The next step is to evaluate this LipL32 real-time PCR assay in the clinical use. The practicability of the method will make it suitable for diagnosis in the early phase of the illness, before antibodies are detectable and several weeks before culture results are available.

Choosing LipL32 as the target gene for detection could help differentiate pathogenic and non-pathogenic Leptospira species. Non-pathogenic Leptospira species are commonly found in environmental samples and frequently confused the identification of pathogenic strains from environmental contaminants. Therefore, LipL32 real-time PCR assay would be applied for the testing of environmental samples.

The prevalence of human leptospirosis is particularly high in warm humid countries (4). As Taiwan's location, between tropical and subtropical regions, with warm and humid climate, and most importantly the diverse animal reservoirs, Taiwan indeed provides suitable conditions for the survival and transmission of Leptospira spp, because leptospires can survive in warm, moist soil and in water for weeks to months. Nevertheless, Chou et al. reports that the annual incidence of probable leptospirosis in Taiwan is 0.21-0.57 case/100,000 population in 2001-2007 (2). The data is extremely low as compared to the data from World Health Organization, the incidence of probable

leptospirosis is 10-100 case/100,000 population/year in humid tropical regions. The data would be underestimated and the main reason may be the difficulty in diagnosis. We believe that by incorporated our real-time PCR assay as an additional diagnostic method in the laboratory will result in a more accurate analysis.

In conclusion, we report on the development of real-time PCR assay using a fluorogenic TaqMan probe with specific detection of pathogenic Leptospira. This assay can detect as few as 10 leptospires per reaction. This method is simple, rapid, and has applications as both research and diagnostic testing in human, veterinary fields, and in the outbreak investigation.

Between May and Sep. 2011, first four cases of human brucellosis in 30 years were confirmed in Taiwan. All cases occurred in returned travelers from regions where brucellosis is endemic. It reminds us of the possible threat of this neglected zoonosis. During the early stages of the disease, patients are frequently bacteremic so circulating brucellae are easily detected by blood culture(14). Therefore all clinical labs may encounter a positive isolate of *Brucella* spp. and need to be aware of its high transmissibility through laboratory procedures. Three of the four cases this year had their blood culture performed in hospital and reported to be positive for *Brucella* spp. resulting in dozens of exposed laboratory personnel. The attack rate of laboratory acquired brucellosis ranged from 30-100% in different reports, and the risk of transmission is not limited to staff who process the isolate (14). Health Protection Agency in the United Kingdom and Centers for Disease Control and Prevention in the United States have proposed guidelines for managing staff with possible exposure to *Brucella* spp (http://www.cdc.gov/nczved/divisions/dfbmd/diseases/brucellosis/recommendations.html,

(8). With different categorization of exposure, the two guidelines both emphasize the importance of serological follow up and post-exposure prophylaxis. Following diagnosing four cases this year, TCDC has proposed their own recommendations recently (http://www.cdc.gov.tw/public/Attachment/18258152971.pdf). Post-exposure prophylaxis and serological follow up will be offered to exposed laboratory staff by TCDC.

Human brucellosis has been listed as the category IV notifiable disease by Taiwan Centers for Disease Control (TCDC) in 2012. Travelers, travel medicine doctors, travel agencies and tour guides should all be familiar with food safety principles and proper protective measures when animal contact is unavoidable. Infectious diseases know no boundaries. As the epidemiology of zoonosis changes with changing lifestyle and evolving human-animal interaction, we should be alert and flexible.

Acknowledgements

This work was supported by a grant-in-aid of Ministry of Health, Labor and Welfare, the Government of Japan (H23-Shinkou- shitei-020).

References

1. **Brown, P. D., C. Gravekamp, D. G. Carrington, H. van de Kemp, R. A. Hartskeerl, C. N. Edwards, C. O. Everard, W. J. Terpstra, and P. N. Levett.** 1995. Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. J Med Microbiol **43:**110-114.
2. **Chou, Y. L., C. S. Chen, and C. C. Liu.** 2008. Leptospirosis in Taiwan, 2001-2006. Emerg Infect Dis **14:**856-857.
3. **Huang XT, W. Y., Tung HP, Chen WC, Su CP, Chang SJ, et al.** 2011. Case report of two imported Brucellosis cases, May 2011. Taiwan Epidemiology Bulletin **27:**5.
4. **Levett, P. N.** 2001. Leptospirosis. Clin Microbiol Rev **14:**296-326.
5. **Levett, P. N., R. E. Morey, R. L. Galloway, D. E. Turner, A. G. Steigerwalt, and L. W. Mayer.** 2005. Detection of pathogenic leptospires by real-time quantitative PCR. J Med Microbiol **54:**45-49.
6. **Merien, F., G. Baranton, and P. Perolat.** 1995. Comparison of polymerase chain reaction with microagglutination test and culture for diagnosis of leptospirosis. J Infect Dis **172:**281-285.
7. **Merien, F., D. Portnoi, P. Bourhy, F. Charavay, A. Berlioz-Arthaud, and G. Baranton.** 2005. A rapid and quantitative method for the detection of Leptospira species in human leptospirosis. FEMS Microbiol Lett **249:**139-147.
8. **Reddy, S., R. Manuel, E. Sheridan, G. Sadler, S. Patel, and P. Riley.** 2010. Brucellosis in the UK: a risk to laboratory workers? Recommendations for prevention and management of laboratory exposure. J Clin Pathol **63:**90-92.

9. **Ribeiro, M. A., A. P. Brandao, and E. C. Romero.** 1996. Evaluation of diagnostic tests for human leptospirosis. Braz J Med Biol Res **29:**773-777.

10. **Seleem, M. N., S. M. Boyle, and N. Sriranganathan.** 2010. Brucellosis: a re-emerging zoonosis. Vet Microbiol **140:**392-398.

11. **Slack, A., M. Symonds, M. Dohnt, C. Harris, D. Brookes, and L. Smythe.** 2007. Evaluation of a modified Taqman assay detecting pathogenic Leptospira spp. against culture and Leptospira-specific IgM enzyme-linked immunosorbent assay in a clinical environment. Diagn Microbiol Infect Dis **57:**361-366.

12. **Smythe, L. D., I. L. Smith, G. A. Smith, M. F. Dohnt, M. L. Symonds, L. J. Barnett, and D. B. McKay.** 2002. A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp. BMC Infect Dis **2:**13.

13. **Trueba, G., S. Zapata, K. Madrid, P. Cullen, and D. Haake.** 2004. Cell aggregation: a mechanism of pathogenic Leptospira to survive in fresh water. Int Microbiol **7:**35-40.

14. **Yagupsky, P., and E. J. Baron.** 2005. Laboratory exposures to brucellae and implications for bioterrorism. Emerg Infect Dis **11:**1180-1185.

Publication list for this work

NIL

Figures and Tables

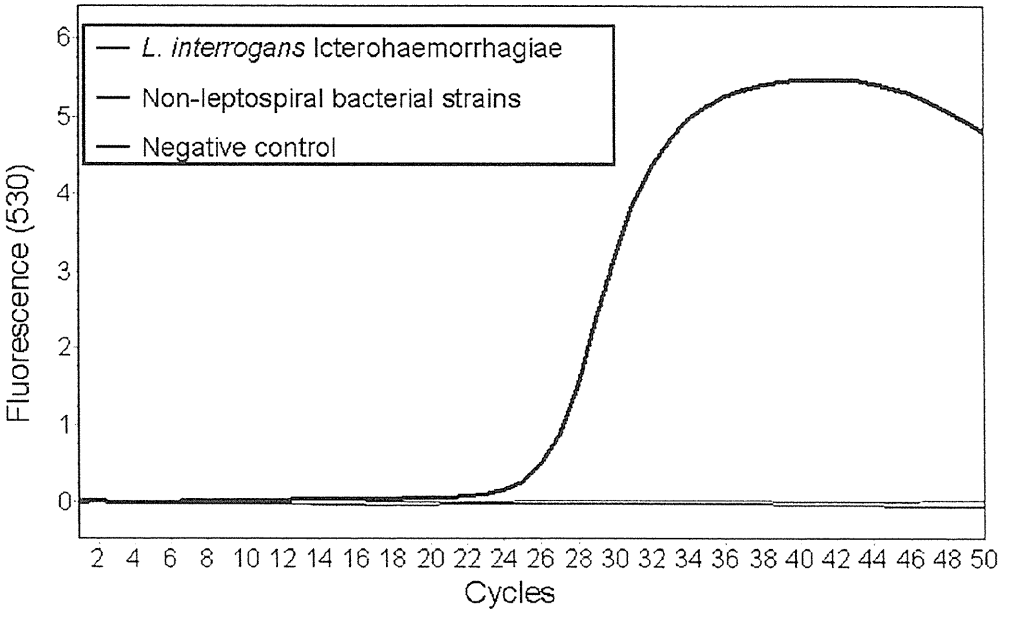

Fig. 1. Specificity of LipL32 real-time PCR

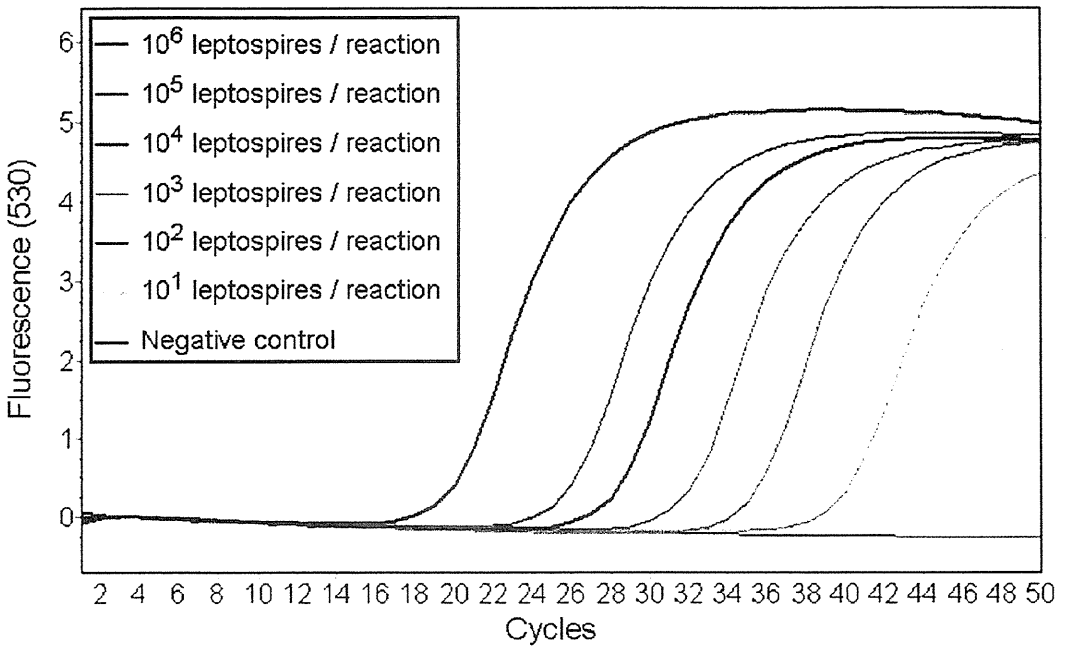

Fig. 2. Sensitivity of LipL32 real-time PCR

Table 1. List of *Leptospira* strains used for LipL32 real-time PCR assay and results after amplification

Serogroup	Serovar	Strain	Genomospecies	Pathogenicity	LipL32 Real-time PCR
Javanica	Poi	Poi	*L. borgpetersenii*	pathogenic	+
Tarassovi	Tarassovi	Perepelistin	*L. borgpetersenii*	pathogenic	+
Canicola	Canicola	Hond Utrecht IV	*L. interrogans*	pathogenic	+
Australis	Bratislava	Jez Bratislava	*L. interrogans*	pathogenic	+
Pomona	Kennewicki	LT1026	*L. interrogans*	pathogenic	+
Icterohaemorrhagiae	Icterohaemorrhagiae	CF-1	*L. interrogans*	pathogenic	+
Autumnalis	Autumnalis	Akiyami A	*L. interrogans*	pathogenic	+
Icterohaemorrhagiae	Copenhageni	No 8 Vcop	*L. interrogans*	pathogenic	+
Pomona	Pomona	Pomona	*L. interrogans*	pathogenic	+
Autumnalis	Djasiman	Djasiman	*L. interrogans*	pathogenic	+
Pyrogenes	Pyrogenes	Izena	*L. interrogans*	pathogenic	+
Kremastos	Kremastos	Kremastos	*L. interrogans*	pathogenic	+
Bataviae	Bataviae	Van Tienen	*L. interrogans*	pathogenic	+
Cynopteri	Cynopteri	3522C	*L. kirscheneri*	pathogenic	+
Panama	Panama	CZ 214	*L. noguchii*	pathogenic	+
Shermani	Shermani	ATCC 43286	*L. santarosai*	pathogenic	+
Lyme	Lyme	ATCC 43289	*L. inadai*	intermediate	-
Semaranga	Patoc	Patoc 1	*L. biflexa*	saprophytic	-