Table 2. Comparison of the Taiwan's GII.4 strains amino acids changes in predicted potential immunoloops within P2 subdomain and HBGA binding pocket. Three immunoloops contain loop1 (position 294-297), loop2 (position 371-374) and loop3 (position 390-393). HBGA binding sites include site 1 (position 343-345, 374) and site 2 (position 393-395). Gray color means the same variation between GII.4 2012 strain and GII.4 2008b strain. Diagonal stands for the variation different to past strains.

	294	295	296	297	305	310	340	341	343	344	345	357	359	364
07-B-1(2006b)	A	S	S	R	S	N	G	D	S	T	R	P	T	S
08-W-1(2008b)	T				T	S	A					D	A	R
09-BI-2-1(2009b)	P					S	T	N				D	S	R
12-AY-1(2010)	P					S	T	N				D	S	R
12-BA-1(2012-Mar)	T						T					D	A	R
12-BQ-1(2012-Jul)	T						T					D	A	R
12-CD-2-4(2012-Aug)	T						T					D	A	R
12-CG-2-4(2012-Sep)	T						T					D	A	R

	368	371	372	373	374	376	377	378	393	394	395	396	413	414
07-B-1(2006b)	S	T	E	N	D	E	T	H	S	T	T	H	V	H
08-W-1(2008b)	A		D			D	A	N	D		A			
09-BI-2-1(2009b)	A		D					N				P	I	
12-AY-1(2010)	A		D					N				P	I	
12-BA-1(2012-Mar)	E		D	H			A	N	G				T	
12-BQ-1(2012-Jul)	E		D	H			A	N	G				T	
12-CD-2-4(2012-Aug)	E		D	H			A	N	G				T	
12-CG-2-4(2012-Sep)	E		D	H			A	N	G				T	

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Development of diagnostic methods for Leptospirosis and Surveillance of canine Brucellosis in Taiwan

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Summary:

Leptospirosis and brucellosis are important zoonotic infections. In the three-year project, we developed the LipL32 real-time PCR for early diagnosis of leptospirosis and also established ELISA method for detecting antibody titer in clinical serum samples. The real-time PCR confirmed the diagnosis of 20 patients (3.3%) from 606 suspected cases of acute leptospirosis with detection limit to 10^3 leptospires/ml in serum. In addition to the molecular method, we also established ELISA methods for detecting antibody against leptospira. LipL32 recombinant protein and leptospira whole cells were used individually as antigens for establishing ELISA methods. Evaluation of these antigens by comparing with MAT titer indicated that Leptospira whole cells to be a potentially useful antigen for detection of leptospirosis. For diagnosis of Brucellosis, serum agglutination test was used to detect human Brucella infection in reported cases and exposed laboratory staff. This method has been employed in the surveillance of canine Brucelosis in the following years. 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.

Purpose:

The main purpose of this study was to develop a rapid diagnostic method of human leptospirosis. Considering the advantage of PCR technology, we choose a TaqMan probe based real-time PCR targeted to LipL32 as our first choice (Brown et al., 1995; Levett et al., 2005; Slack et al., 2007). The specificity and sensitivity of our real-time PCR assay has been demonstrated to a satisfactory condition in the report of second year. We tried to employ the potential of LipL32 real-time PCR assay for the detection in clinical samples. Since this real-time PCR only suitable for diagnosis in the early phase of the illness (Levett et al., 2005; Merien et al., 1995), most of the leptospirosis cases were confirmed by using antibody detection during several weeks after disease onset. Therefore, in the third year project, we established an ELISA method to detect leptospira antibody against LipL32 or the leptospira whole cells, respectively (Ribeiro et al., 1996).

Human brucellosis has been listed as the category IV notifiable disease by Taiwan Centers for Disease Control (TCDC) in 2012. The microplate agglutination test improved diagnosis of human brucella infection in Taiwan and could help to establish surveillance system of brucellosis. Since canine brucellosis is a zoonotic disease that can lead to canine reproductive losses and human infection through contact with infected urine or other genitourinary secretions. Therefore, we used the microplate agglutination test for detection of antibody against Brucella canis from domestic and stray dogs in the third year. This method will be useful for surveillance of canine Brucellosis in Taiwan.

Methods:

1) **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.

2) **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 master mix, 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 (LipL32-F (5'-GAAATGGGAGTTCGTATGATTTCC-3', LipL32-R (5'-GGTTTTGCTTTCGCAGCTT-3') and 200 nM of the TaqMan probe (5'FAM-TAATCGCCGACATTCTTTCTACACGGATC-3'BHQ). 10 uL of PCR master mix and 10 uL of DNA extract were loaded into a LightCycler capillary and centrifuged to mix. 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. PCR will result in a 194 bp amplicon between position 274 and 467 of the LipL32 coding region.

3) **LipL32 and Leptospira whole cell ELISA method**

The cloning and expression of recombinant LipL32 were derived from Dr. Hunag CC (Yang et al., 2002). For coating plates with recombinant LipL32 antigen, the purified antigen was diluted with bicarbonate buffer and put into 96 well microplates for incubation at 4 °C. The second day, discard diluent and add blocking solution, stored at 4 °C until used. For coating with leptospira whole cells, the L. santarosa serotype shermanni was cultured in EMJH broth for 10-12 days at 28°C. After havesting, the leptospire culture was killed with formalin (0.5% final concentration) for 1 hour. The leptospire culture was boiled for 30 min and centrifuged for 30 min at 10,000 rpm. The supernatant was kept as whole cell lyste antigen and put into 96 well microplates. The plates were left at room temperature until complete evaporation of fluid.

Sera were made for 80X dilution. Positive serum was made two-time series dilution from 200X to 25600X. Adding 100 ul sera into each microwell and incubated at 37 °C for 30 min. Washing 3 times, then adding 6000X anti-human IgM HRP-conjugated secondary antibody and incubated at 37 °C for 30 min. Washing 3 times, then adding TMB solution for 4 min. Adding TMB-stopping solution and read at 450 nm. Dividing the highest OD of positive sera by 2, this is X. All sera dilutions with an OD higher than X were considered positive.

4) **Microplate Agglutination Test for canine Brucellosis**

Blocking microplates wells with 125 ul of milk or BSA Incubate at room temperature for 1hr and then discard blocking solutions. Positive and negative sera were incubated with heat-inactivated *B. canis* strain QE-13 whole cells in a U-bottom 96 well culture plate. The sealed plate were mixed and incubated at 50℃ for 24 hr in a humid atmosphere. An agglutination titer greater than 160 was considered positive.

Results:

1) **Detection of leptospiral DNA in human clinical samples.**

The real-time PCR established in the second year was use to confirm acute leptospirosis. Twenty out of 606 reported cases were test positive by real-time PCR with detection limit to 10^3 leptospires/ml in serum. Among these 20 positive cases, MAT seroconversion was observed in fourteen patients (Table 1), no convalescent serum samples were available for 5 patients and no antibody titer rising for 1 patient. Since the PCR signal was invariably positive for the first blood sample, giving unequivocal confirmation of acute leptospirosis. This finding indicates the PCR is suitable for early detection of leptospires in blood.

2) **Evaluation of ELISA coating with LipL32 and Leptospira whole cell for**

possible diagnostic kit for leptospirosis

In addition to the molecular method, we also established ELISA methods for detecting leptospira infection. His tagged LipL32 recombinant protein and leptospira whole cells were used individually as antigens for establishing ELISA methods. Seventy-two serum samples were chosen for detection of antibody using the ELISA method. MAT titer was employed to evaluate the sensitivity and specificity by comparing with coating His-LipL32 or whole cells in the ELISA. The ELISA coating with whole cells presented better both sensitivity and specificity than that coating with His-LipL32 (Table 2). The data indicated that Leptospira whole cells could be a potentially useful antigen for detection of leptospirosis.

3) **Canine Brucellosis surveillance in domestic and stray dogs**

In the third year, we employed Microplate Agglutination Test for canine Brucellosis surveillance. Sixty-three serum samples from 25 domestic and 38 stray dogs were collected. Only one serum sample from stray dog tested positive. Therefore, there is 2.7 % positive rate of canine Brucellosis in stray dogs, and 1.6 % of positive rate in all samples (Table 3).

Discussions:

The LipL32 real time PCR method can specifically detect pathogenic leptospires in the limit of 10 leptospires/ml. Using the real-time PCR to detect leptospires in acute phase serum samples demonstrated 3.3% of Leptospirosis positive rate, increase about 1% of positive rate when compared with antibody detection (Microscopic Agglutination Test, MAT) (Levett et al., 2005). Therefore, the real-time PCR may suitable for detecting samples from outbreak patients (most in acute phase) or reservoir host in the field surveillance. Meanwhile, the establishment of ELISA for leptospirosis diagnosis may apply in the preliminary screen of leptospira infection. The ELISA coating with whole leptospira cells showed better sensitivity and specificity than coating with His tagged recombinant LipL32

Serum agglutination test for Brucellosis has been applied for clinical diagnosis. In the canine Brucellosis surveillance, there is 1.6 % of positive rate, lower than Japan and some countries (Kimura et al., 2008; Lopez et al., 2009). The low positive rate may reflect the fact that human and animal Brucellosis have been eradicated in Taiwan for more than 30 years. However, the samples from stray dogs were heart blood from post-mortem samples, most of them are heavily hemolysed, whether the heavy hemolysis or drugs in post-mortem samples affect the detection will need to be clarified.

In conclusion, we report the development of real-time PCR assay using a fluorogenic TaqMan probe for highly specific detection of pathogenic Leptospira in clinical samples. This method is simple, rapid, and would be helpful in acute phase diagnosis. The ELISA coating with whole leptospira cells showed better result than that with recombinant protein and demonstrated to be a possible method for preliminary screen of Leptospirosis. For the Brucellosis diagnosis, the microplate agglutination test for antibody against Brucella spp. has been applied in clinical use

and also in surveillance. We hope both methods for detection of Leptospirosis and Brcellosis would extend to veterinary fields, and outbreak investigation in the future.

Table 1:LipL32 real-time PCR assay and serologic results from 20 positive patients

			Acute serum		Convalescent serum			(leptospires/ml)
Pt. No.	Sex	Age (years)	Days of sampling	MAT titers	Days of sampling	MAT titers	Main serogroup	LipL32 real-time PCR assay
1	F	87	9	<100	25	200	Shermani	5.90×10^3
2	M	59	5	<100	NA	NA	NA	6.53×10^3
3	M	63	1	<100	NA	NA	NA	6.00×10^3
4	F	65	10	<100	NA	NA	NA	5.20×10^3
5	M	32	4	<100	10	3200	Shermani	1.00×10^5
6	M	47	10	<100	20	<100	<100	8.26×10^3
7	M	50	5	<100	15	3200	Shermani	5.06×10^4
8	M	64	5	<100	21	800	Shermani	1.60×10^5
9	M	71	4	<100	14	6400	Shermani	6.46×10^4
10	M	71	4	<100	14	6400	Shermani	1.07×10^4
11	M	54	3	<100	14	1600	Shermani	5.83×10^3
12	M	41	4	<100	21	3200	Shermani	1.40×10^4
13	M	43	6	<100	17	1600	Shermani	8.50×10^3
14	M	26	3	<100	17	1600	Shermani	2.91×10^3
15	M	42	1	<100	15	6400	Shermani	1.31×10^3
16	M	62	2	<100	15	3200	Shermani	1.00×10^4
17	M	33	5	<100	13	12800	Kennewicki	5.00×10^3
18	M	52	0	<100	NA	NA	NA	2.83×10^3
19	F	61	5	<100	14	1600	Shermani	3.36×10^3
20	M	35	0	<100	NA	NA	NA	1.59×10^4

Table 2:#Establishment of ELISA for screen of Leptospirosis cases

ELISA coating with LipL32

test	MAT confirmed		
	present	absent	total
positive	47	5	52
negative	6	14	20
total	53	19	

ELISA coating with whole cells

test	MAT confirmed		
	present	absent	total
positive	51	2	53
negative	2	17	19
total	53	19	

Table 3: Surveillance of canine Brucella antibody in domestic and stray dogs

Stray dogs		Domestic dogs		Total	
Negative	Positive	Negative	Positive	Negative	Positive
37	1	25	0	62	1
	2.7%		0%		1.6%

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台湾におけるブルセラ症－33 年ぶりの患者報告と届出疾患へ－
国立感染症研究所　獣医科学部　第 1 室　今岡浩一、鈴木道雄
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Genetic diagnosis and molecular epidemiology of *Bordetella*

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Summary:

Bordetella holmesii infection has never been reported in Taiwan. In current surveillance study, only one case of possible *B. holmesii* infection was found among 653 notified cases of pertussis (0.15%) in 2011 - 2013. This possible case was a 12-year-old boy who was notified in May 2011. According to this surveillance results, the prevalence of *B. holmesii* infection in Taiwan was extremely low among patients who had pertussis-like symptoms and were notified. Based on its high sensitivity, the IS*481*-based PCR was routinely used worldwide by many laboratories, however, it should be done with the awareness of its cross-reactivity. *B. holmesii* has been more and more frequently detected in biological samples from adolescents and adults of pertussis-like symptoms. Although differentiation might not be necessary for treatment, it is valuable in epidemiological settings to distinguish among *Bordetella* species. Improved specificity of detection would provide us an insight into the real burdens of *B. pertussis* and *B. holmesii* infection, and information regarding vaccine failure due to misdiagnosis and possible response strategy. In conclusion, surveillance of *B. holmesii* should be pursued, and correct identification of *Bordetella* species is important for active surveillance of *Bordetella* infections in the whole population, particularly in adolescents and adults.

I. Purpose:

In previous surveillance studies conducted in the USA and Canada, a low positive rate for *B. holmesii* infection (0.1–0.3%) was reported by culture or RT-PCR in patients with coughs (1, 2). In a recent study in France, however, *B. holmesii* DNA was detected in 20% of nasopharyngeal swabs (NPSs) collected from adolescent patients who had previously been diagnosed with *B. pertussis* infection (3). Furthermore, between 2010 and 2011, a pertussis outbreak caused by *B. pertussis* and *B. holmesii* infections occurred in Miyazaki Prefecture, Japan (4). These surveillance data indicated that *B. holmesii* infection has recently spread worldwide and that

accurate diagnosis is needed to distinguish between *B. holmesii* and *B. pertussis* infections. Therefore, the purpose of this surveillance study is to detect *B. holmesii* in NPSs received in our laboratory from all cases who had pertussis-like symptoms and were notified in 2011 -2013.

II. Methods:

We applied *B. pertussis*-LAMP assay and a novel duplex real-time PCR assay to NPSs from 653 patients who had pertussis-like symptoms and were notified in 2011 - 2013. These patients were collected from Taiwan pertussis notified disease surveillance system. We obtained the NPSs using ESwab™ Nylon Flocked Swab and 1mL of modified Liquid Amies (Copan).

B. pertussis-LAMP assay (detection of *ptxP*): A 25μL reaction mixture containing 40 pmol (each) of BP-FIP and BP-BIP primers, 5 pmol (each) of BP-F3 and BP-B3 primers, 20 pmol (each) of BP-LF and BP-LB primers, 2X reaction mixture (12.5 μL), *Bst* DNA polymerase (1 μL), and template DNA (2 μL) was used. The mixture was incubated at 65°C for 40 min (for clinical specimens) and then heated at 80°C for 2 min to terminate the reaction. All oligonucleotides (high-performance liquid chromatography purification grade) for the LAMP primers were obtained from Invitrogen Taiwan Ltd. The LAMP amplification was confirmed with real-time monitoring of the increase of turbidity using LA-320C (Eiken Chemical Co., Ltd.) (5).

Novel duplex real-time PCR assay (detection of IS*481* and BH*recA*): The duplex PCR master mix consisted of 1X Premix master mix (Premix EX Taq, RR039A, Takara), 0.8 μM (each) BH*recA* forward and reverse primers, 0.4 μM BH*recA* probe, 1 μM (each) IS*481* forward and reverse primers, 0.25 μM IS*481* probe, 2 μL of template DNA, and enough sterile nuclease-free water to bring the total reaction volume to 20 μL. The samples were subjected to an initial amplification cycle of 95°C for 30s, followed by 40 cycles at 95°C for 5s and 60°C for 34s. Two microliters of *B. holmesii* ATCC51541 (500 pg/μL) with 6 series of 10-fold dilution was used as a positive PCR control; the negative control was 2 μL of sterile H_2O. Amplification, detection, and data analysis were performed with an Applied Biosystems 7500 real-time PCR system and the 7500 software v2.0 (1).

III. Results:

1) Results of real-time PCR using IS*481*, BH*recA* and *ptxP* (LAMP) for NPSs in 2011-2013

Target	Target organism	2011		2012		2013	
		No. of specimens	% of total specimens	No. of specimens	% of total specimens	No. of specimens	% of total specimens
IS*481* without BH*recA*	*B. pertussis*	60	16.7	32	22.4	34	22.6
IS*481* and BH*recA*	*B. holmesii*	1	0.3	0	0	0	0
ptxP (LAMP)	*B. pertussis*	47	13.1	27	18.9	32	21.2
Total specimens tesed		359		143		151	

B. holmesii DNA was detected only in one case notified in May 2011. This case was a 12-year-old boy. Positive rate of *B. pertussis* using IS*481*-based PCR was 16.7%, 22.4% , 22.6% in 2011-2013, respectively. Positive rate of *B. pertussis* using *ptxP*-based PCR was 13.1% , 18.9%, 21.2% in 2011 - 2013, respectively.

2) An investigation of a school outbreak of pertussis

In December 2013, a suspected outbreak of pertussis occurred in a middle school in Longtan, Taoyuan County. The index case was a 13-year-old boy. He started with flu-like symptoms on November 10, and went to the En Chu Kong Hospital for medical service on December 10 due to persistent symptoms. He was notified as a case with pertussis. He was tested positive for pertussis by PCR on December 17, and by bacterial culture on December 19. A school clustering of pertussis was found through the System of Epidemiological Investigation at the Centers for Disease Control. Nasopharyngeal swabs were obtained from 8 contacts who developed pertussis-like symptoms between November 27 and December 9. Four contacts were tested positive for pertussis by *ptx*P-based PCR, and IS*481* without HB*recA* real-time PCR on December 20, and later by bacterial culture.

IV. Discussion:

Surveillance result of *B. holmesii* infection in Taiwan revealed that there was only one possible case, a 12-year-old boy, in May 2011. This result indicated that the prevalence of *B. holmesii* infection in Taiwan was very low among patients who had pertussis-like symptoms and were notified. Whether this case was a real case of *B. holmesii* infection was not certain. Although the duplex real-time PCR gave a positive result, sequencing of the DNA product was not successful because the *B. holmesii* DNA content in the specimen was too low.

The positive rate of diagnosis was 50% among the investigation of the school outbreak of pertussis, revealing that pertussis is a highly contagious disease. Following the confirmation of the index case, all contacts with pertussis-like symptoms received antimicrobial agents for 5 days. No more cases were recognized then, indicating that rapid, accurate diagnosis combined with correct treatment and prevention will greatly aid in disease control. The duplex real-time PCR is the method that can provide timely and accurate diagnosis to reveal pathogens that cause the infections.

From studies worldwide, most cases from whom *B. holmesii* was detected were adolescents and adults, especially significant occurrence in adolescents, but not in infants. However, the age distribution of our cases was 55.1% , 48.5% , 68.9% among infants less than 1 year old in 2011 - 2013, respectively, and 12.7% , 12.5%, 9.9% among children aged 10-19 years in 2011 - 2013, respectively. There might not be sufficient specimens from adolescents in our study, thus, leading to low prevalence. Nevertheless, *B. holmesii* was indeed present and associated with pertussis-like symptoms in patients, indicating that surveillance of *B. holmesii* infection is important.

Based on its high sensitivity, the IS*481*-based PCR was routinely used worldwide by many laboratories, however, it should be done with the awareness of its cross-reactivity. *B. holmesii* has been more and more frequently detected in biological samples from adolescents and adults of pertussis-like symptoms. Although differentiation might not be necessary for treatment, it is valuable in epidemiological settings to distinguish among *Bordetella* species. Improved specificity would advance our understanding of burdens from *B. pertussis* and *B. holmesii*, reduce concerns arising from apparent vaccine failures due to misdiagnosis, and might provide information on which vaccine-based outbreak response strategies can be based (6). In conclusion, surveillance of *B. holmesii* should be pursued and correct identification of *Bordetella* species is important for active surveillance of *Bordetella* infections in the whole population, particularly in adolescents and adults.

V. Reference list:

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VI. Publication list for this work:

Nil