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 台湾行政院衛生署疾病管制局　　研究検験中心　細菌実験室　慕蓉蓉