Development of diagnostic methods for brucellosis and leptospirosis

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Summary:

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 epidemiology of Brucellosis, we tried to conduct a serum surveillance of canine Brucellosis using microplate agglutination test to detect antibody in stray and household dogs. These techniques improved diagnosis of leptospira and brucella infection and could help to understand the epidemiology of leptospirosis and brucellosis in Taiwan in the future.

I. Purpose:

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 (16, 19, 23, 24). Meanwhile using this real-time PCR assay in 606 plasma samples, 20 samples (3.3%) were positive (average 4.3 days after disease onset), and 14 of 20 showed seroconversion at the convalescent serum samples (average 16.1 days after disease onset). Our experimental data also demonstrated the potential of LipL32 real-time PCR assay for the detection in clinical samples and the subsequent quantification in a single run. The practicability of the method makes it suitable for diagnosis in the early phase of the illness, before antibodies are detectable and several weeks before culture results are available.

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. In this report, we fine-tuned the microplate agglutination test for detection of antibody against

Brucella canis among dogs. This method will apply for surveillance of canine Brucellosis in Taiwan.

II.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**

3) 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.

4) Microplate Agglutination Test for canine Brucellosis

   Blocking microplates wells with 125ul 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.

III. Results:

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

   We have established LipL32 real-time PCR assay for detection of leptospira DNA in the first year. To evaluate the LipL32 real-time PCR assay as a potential tool for detection from clinical samples, 606 patient sera were examined by the LipL32 real-time PCR and the positive cases were re-confirmed by either nested PCR or MAT. Twenty plasma samples were tested positive by LipL32 real-time PCR assay with concentration from

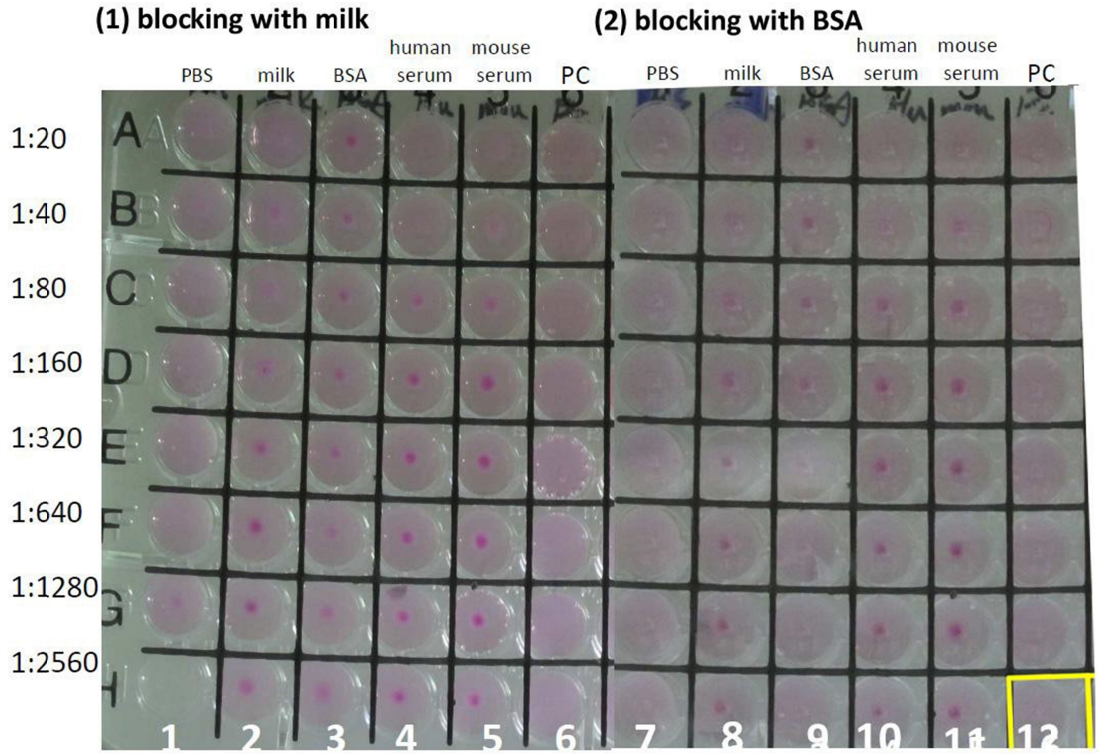
1.60 x 10^5 to 1.31 x 10^3 leptospires/mL of sera. Among the 20 positive cases, MAT seroconversion was observed in fourteen patients (Table 4), no convalescent serum samples were available for 5 patients and no antibody titer rising for 1 patient. Since the PCR signal was invariably positive for the first blood sample, giving unequivocal confirmation of acute leptospirosis. This finding indicates the PCR is suitable for early detection of leptospires in blood.

Table 4. LipL32 real-time PCR assay and serologic results from 20 patients Days of sampling

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

2) **Fine-tuned microplate agglutination test for antibody detection of B. canis**

Low protein concentration of samples, especially highly diluted ones did not show typical negative pattern (Table 2, columns 1 and 7). Therefore, blocking wells with milk and BSA was used to improve the method (Table 2, columns 2, 8 and 3, 9). Human and mouse serum were used as negative control (Table 2, columns 4,10 and 5, 11) and showed improved results. Blocking with both milk and BSA did not affect the positive control. (Table 2, columns 6 and 12).



IV.Discussion:

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 assay can detect as few as 10 leptospires per reaction. This method is simple, rapid, and has applicated for diagnostic testing in clinical specimen. The microplate agglutination test for antibody against Brucella canis was modified and ready to apply for detection canine sera. We hope both methods can extend to veterinary fields, and outbreak investigation in the future.

V.Reference list:

NIL

VI.Publication list for this work:

NIL