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Optimization of *LipL32* PCR assay for increased sensitivity in diagnosing leptospirosis

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Abstract

Early diagnosis of leptospirosis in humans is critical with regard to initiation of appropriate treatment; however, the gold standard serological test cannot detect antibodies until nearly a week after symptom onset. PCR has been shown to be sensitive and specific in the early phase of leptospirosis. Previously, we developed and validated a TaqMan PCR assay targeting *lipL32*. We reoptimized and validated this assay using PerfeCTa® qPCR ToughMix®, Low ROX™ (Quanta Biosciences, Gaithersburg, MD, USA). For optimization with the new mix, the final primer concentrations were increased from 0.5 μmol/L to 0.9 μmol/L compared to our previous assay, and the probe concentration increased from 0.1 μmol/L to 0.125 μmol/L. This newly optimized assay resulted in a lower limit of detection and increased diagnostic sensitivity. Here, we present the performance data of the improved assay and describe several clinical cases that were initially negative but tested positive using the optimized assay.

Keywords

Leptospirosis; Diagnosis; Real time PCR

Early diagnosis of leptospirosis in humans is critical with regard to initiation of appropriate treatment; however, the gold standard serological test cannot detect antibodies until nearly a week after symptom onset (Levett, 2001). PCR has been shown to be sensitive and specific in the early phase of leptospirosis (Ahmed et al., 2009; Slack et al., 2007; Stoddard, 2013). Previously, we developed and validated a TaqMan PCR assay targeting *lipL32* (Stoddard, 2013). We reoptimized and validated this assay using PerfeCTa® qPCR ToughMix®, Low ROX™ (Quanta Biosciences, Gaithersburg, MD, USA). For optimization with the new mix, the final primer concentrations were increased from 0.5 μmol/L to 0.9 μmol/L compared to our previous assay, and the probe concentration increased from 0.1 μmol/L to 0.125 μmol/L. This newly optimized assay resulted in a lower limit of detection and increased diagnostic sensitivity. Here, we present the performance data of the improved assay and describe several clinical cases that were initially negative but tested positive using the optimized assay.

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DNA was extracted from 2 genetically distinct strains of *Leptospira (Leptospira interrogans* serovar Icterohaemorrhagiae strain RGA and *Leptospira borgpetersenii* serovar Ballum strain Mus 127). Serial dilutions of genomic DNA starting at 1×10^5 GE/μL down to 1×10^{-1} GE/μL were made. Three replicates of each dilution were assayed by PCR on 3 separate days using the original and newly optimized assays. The lower limit of detection at which 100% of the replicates yielded a positive reaction was 5×10^1 GE/μL and 5×10^2 GE/μl for the *L. interrogans* and *L. borgpetersenii* strains, respectively, using the original assay (5 μL of DNA was added to each PCR reaction). Using the updated assay, the lower limit of detection decreased to 5×10^0 GE/μL and 5×10^1 GE/μL for each strain, respectively (Table 1).

The same 2 strains of *Leptospira* were used to spike donor blood, sera, and urine at concentrations ranging from 1×10^6 to 1×10^1 organisms/mL. Concentrations were determined by enumerating *Leptospira* organisms using a Petroff–Hausser counting chamber. Each concentration in each matrix was extracted 3 times on different days. Three replicates of each extraction were tested in the PCR reaction using both the original and the optimized assay conditions. The newly optimized assay improved the lower limit of detection in blood for the *L. interrogans* strain and remained equal to the original assay for detection in serum and urine (Table 1). For the *L. borgpetersenii* strain, the optimized assay improved detection in blood and serum and remained equal to the original assay in urine.

Clinical samples submitted for leptospirosis PCR were run in parallel using both formats during the validation phase of the optimized assay. Several cases were negative using the original assay but positive with the optimized assay (Table 2). Case 3 showed signs of PCR inhibition using the original assay with the cerebral spinal fluid (CSF) specimen only, as the exogenous DNA that is added to the master mix as an internal positive control was negative. The PCR was repeated using a 1:10 dilution of the DNA extracted from the CSF, which presumably diluted the inhibitor and then gave a positive PCR result. The optimized assay was unaffected by inhibitors and gave a positive result undiluted. We also observed PCR inhibition with samples extracted by an automated Promega Maxwell 16 instrument with the use of the original assay, but not with the optimized assay (data not shown). The optimized assay appears to be less affected by PCR inhibitors that may be present in clinical specimens or by using certain extraction methods.

The optimal specimen for our PCR assay is acute whole blood (Stoddard, 2013). The organisms are detectable in the bloodstream only for a transient period of time before they sequester into tissues (Levett, 2001). Generally speaking, once there is a detectable immunological response, the PCR assay is negative. Therefore, some convalescent serum specimens in Table 2 were not tested using the PCR assay. Additionally, these clinical results reinforce the importance of using acute samples for diagnostic PCR testing, with the exception of case 5, which had some special circumstances (Wilson et al., 2014). This patient had symptoms of meningitis upon his third hospitalization after initially presenting with uveitis and was likely a rare instance of chronic neuroleptospirosis in a patient with an immune deficiency.

In conclusion, our optimized PCR assay exhibited a lower limit of detection and was more sensitive on donor specimens spiked with 2 genetically distinct strains of *Leptospira*. This assay was also more sensitive on real specimens in the highlighted cases tested to date and played a critical role in confirming the diagnosis of leptospirosis.

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Table 1

Limits of PCR detection in different matrices for 2 genetically distinct strains of *Leptospira* for both the old and new assay.

		Original PCR assay	Optimized PCR assay
L. interrogans serovar Icterohaemorrhagiae	Culture	5×10^1 GE/μL	**5×10^0 GE/μL**[a]
	Blood	1×10^3 leptospires/mL	**1×10^2 leptospires/mL**
	Serum	1×10^4 leptospires/mL	1×10^4 leptospires/mL
	Urine	1×10^4 leptospires/mL	1×10^4 leptospires/mL
L. interrogans serovar Ballum	Culture	5×10^2 GE/μL	**5×10^1 GE/μL**
	Blood	1×10^3 leptospires/mL	**1×10^1 leptospires/mL**
	Serum	1×10^4 leptospires/mL	**1×10^3 leptospires/mL**
	Urine	1×10^4 leptospires/mL	1×10^4 leptospires/mL

[a]Bold text indicates improved detection over the original assay.

Table 2

Comparison of the original and optimized assays using clinical specimens submitted for testing.

Case	Specimens submitted for testing	Original PCR result	Optimized PCR result	Serology results	Case description	Days post symptom onset
Case 1	**Serum**[a]	Negative	**Ct 37.3**	MAT[b] negative	25-year-old male from Ohio, swam in reservoir with case 2, hospitalized in intensive care unit.	4
	Serum	Negative	Negative	MAT negative		5
	Whole blood	Negative	**Ct 35.2**	Not applicable		6
	Whole blood	Negative	Negative	Not applicable		7
	Whole blood	Negative	Negative	Not applicable		8
	Urine	Negative	Negative	Not applicable		8
Case 2	**Whole blood**	Negative	**Ct 34.3**	Not applicable	23-year-old male from Ohio, also swam in reservoir with case 1. Fever and body aches, received fluids.	4, AM draw
	Whole blood	Negative	**Ct 36.0**	Not applicable		4, PM draw
	Serum	Negative	**Ct 36.0**	MAT negative		4, PM draw
	Whole blood	Negative	Negative	Not applicable		6
	Urine	Negative	Negative	Not applicable		6
	Whole blood	Negative	Negative	Not applicable		7
	Serum	Not tested	Not tested	**MAT titer 1:25,600**		20
Case 3	**CSF**	33.5[c]	35.0	Not applicable	17-year-old male from Iowa with meningitis, swam in river.	7
	Urine	Negative	Negative	Not applicable		9
	Whole blood	Negative	Negative	Not applicable		10
Case 4	**Whole blood**	Negative	**Ct 36.0**	Not applicable	23-year-old male from Hawaii with history of water exposure.	5
	Serum	Negative	Negative	**MAT titer 1:400**		6
	Whole blood	Negative	Negative	Not applicable		18
	Serum	Negative	Negative	**MAT titer 1:25,600**		18
Case 5	**CSF**	Negative	34.4	Not applicable	14-year-old with history of travel to Puerto Rico. Positive for leptospirosis using next-generation sequencing (Wilson et al., 2014).	140
	Serum	Negative	Negative	Negative[d]		190

[a] Bold text indicates positive results.

[b] Microscopic Agglutination Test.

[c] Specimen was diluted 1:10 due to PCR inhibition.

[d] Serology may have been negative due to an immune deficiency.