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Performance of electrochemical aptasensor as antigen test in clinical samples for early diagnosis of leptospirosis

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Early diagnosis of leptospirosis is critical for timely treatment and effective disease management. This study evaluated the diagnostic performance of a novel electrochemical aptasensor targeting the electron transfer flavoprotein subunit beta (EtfB) of *Leptospira interrogans* in clinical samples collected during the acute phase of leptospirosis. The aptasensor assay was tested using plasma samples and compared to the microscopic agglutination test (MAT), the standard reference method. To assess diagnostic performance, aptasensor results were evaluated against leptospirosis status as determined by MAT. Receiver operating characteristic (ROC) analysis identified a 40% decrease in electrochemical signal relative to the blank as the optimal cut-off, yielding an area under the curve (AUC) of 0.93. The assay demonstrated a sensitivity of 100% and a specificity of 80%. For diagnostic concordance, aptasensor results were compared with those obtained from the reference quantitative PCR (qPCR) method. The aptasensor exhibited 100% positive agreement and 57.1% negative agreement with qPCR. Notably, in patients with high MAT titers, the aptasensor outperformed qPCR in detection rates (100% vs. 25%). These findings indicate that the aptasensor assay is a highly reliable and effective antigen-based diagnostic tool for early leptospirosis detection, making it suitable for both low- and high-prevalence settings.

Keywords Early diagnosis, Leptospirosis, Antigen test, Diagnostic performance, Aptamer biosensor, Aptasensor

Leptospirosis is a globally significant zoonotic disease characterized by a wide spectrum of clinical symptoms, ranging from mild, non-specific febrile illness to severe complications such as renal failure, hepatic dysfunction, and pulmonary hemorrhage[1–4]. This broad range of symptoms makes leptospirosis particularly challenging to diagnose, especially in its early stages, as its clinical manifestations often overlap with those of other infectious diseases, including dengue fever, malaria, and influenza[2,5].

Diagnosis of leptospirosis can be achieved through direct or indirect methods. Direct methods involve the detection of leptospires using techniques such as microscopy, bacterial culture, polymerase chain reaction (PCR), and serology[6,7]. Conventional serodiagnosis relies on detecting antibodies against leptospires through methods like the microscopic agglutination test (MAT), indirect immunofluorescence, and enzyme-linked immunosorbent assay (ELISA)[3,6,7]. However, these approaches are often delayed, as antibodies typically become detectable in the blood only 5–7 days after symptom onset. Furthermore, while PCR is a highly effective diagnostic tool, it is relatively expensive and requires specialized laboratory equipment. Therefore, there is a

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pressing need to develop alternative, advanced diagnostic tests to improve the timely and accurate detection of leptospirosis.

Early and accurate diagnosis is crucial for effectively managing and treating leptospirosis and current diagnostic methods, including serology and molecular techniques, still have significant limitations. There is an urgent need for diagnostic methods capable of providing early, accurate, and easy-to-perform detection of leptospirosis. One promising approach is antigen detection, which can potentially identify leptospiral components in patient samples directly. Antigen detection can facilitate early diagnosis, particularly in the acute phase of the disease when antibodies may not be detectable. Moreover, antigen detection methods can be designed for point-of-care use, making them invaluable in resource-limited settings where leptospirosis is most prevalent.

Developing an antigen detection test for early disease diagnosis requires specific antibodies recognizing distinct antigens in microorganisms. In general, monoclonal antibody manufacturing is widely employed. Recently, aptamers have gained attention as another recognition component for detecting proteins[8–11]. Aptamers and antibodies share many characteristics in terms of antigen recognition and binding, and aptamers are sometimes referred to as "chemical antibodies" or antibody substitutes[12]. However, aptamers offer many advantages over antibodies: they are smaller in size, easily modifiable, more cost-effective to produce, and can be generated to target a broad range of molecules[13,14].

In previous work, we developed a nuclease-resistant RNA aptamer targeting the electron transfer flavoprotein subunit beta (EtfB) of *Leptospira interrogans*, a candidate protein for diagnosis leptospirosis[8]. This aptamer, designated ETFB3-63, was incorporated into an electrochemical aptasensor for EtfB detection. The aptasensor demonstrated satisfactory sensitivity and specificity for detecting EtfB in recombinant and native forms. However, its diagnostic performance has not yet been evaluated using clinical samples. Thus, this study aimed to assess the utility of the aptasensor in differentiating between leptospirosis and non-leptospirosis patients, as verified by the MAT, using plasma samples. Additionally, the diagnostic performance of the EtfB aptasensor was compared with that of the reference PCR method[15] to evaluate its effectiveness for early detection of leptospirosis.

Results

Standard curve for estimating EtfB concentration

A standard curve for estimating EtfB concentration was generated using serial dilutions of recombinant 6xHis-EtfB protein, ranging from 50 to 1000 nM (1.5 to 29.0 µg/mL). The percentage of the signal decrease relative to the blank current was plotted against the ETFB concentrations. The standard curve showed promising results with a good regression ($R^2 = 0.982$) using a four-parameter logistic regression model (Fig. 1A). However, a linear regression ($R^2 = 0.956$) was observed when concentrations ranging from 3.0 to 13.0 µg/mL were analyzed (Fig. 1B).

Optimal cut-off value determination by ROC analysis

The ROC analysis was conducted to evaluate the diagnostic performance of the aptasensor assay and to determine the optimal cut-off value. The ROC curve of the percentage decrease in signal relative to blank (Fig. 2A) demonstrated an AUC of 0.93 (95% CI: 0.84 – 1.00, $p < 0.0001$), indicating high diagnostic accuracy.

Coordinated and customized points from the ROC curve were analyzed in relation to leptospirosis cases to determine the optimal cut-off value. The cut-off value was identified as a percentage signal decrease relative to the blank, based on consistent sensitivity and specificity values derived from coordinated (36.3%) and customized (40%) points (Supplementary Fig. 1). Among these, the percentage decrease in the signal of 40% relative to the blank was established as the optimal cut-off value, yielding an area under the curve (AUC) of 0.90, which was higher than other evaluated thresholds. These findings validate the 40% signal decrease as the optimal threshold

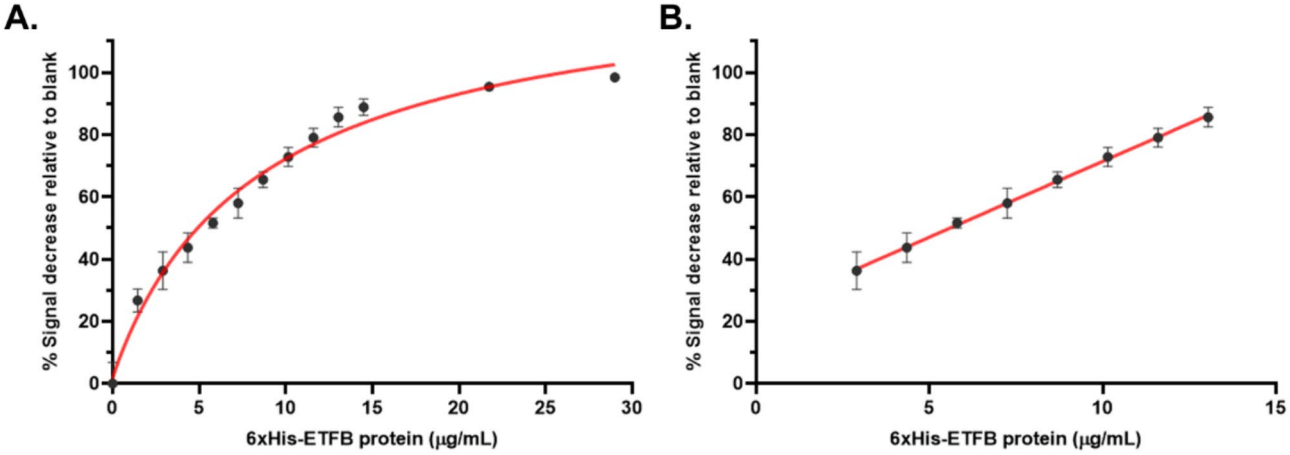

Fig. 1. The calibration curve between the concentration of 6xHis-ETFB and the percentage of the signal decreases relative to the blank. (**A**): The plot fitted with 4-parameter logistic regression ($R^2 = 0.982$). (**B**): The plot fitted with a linear regression ($R^2 = 0.956$) with the slope values $y = 4.8682x + 22.72$.

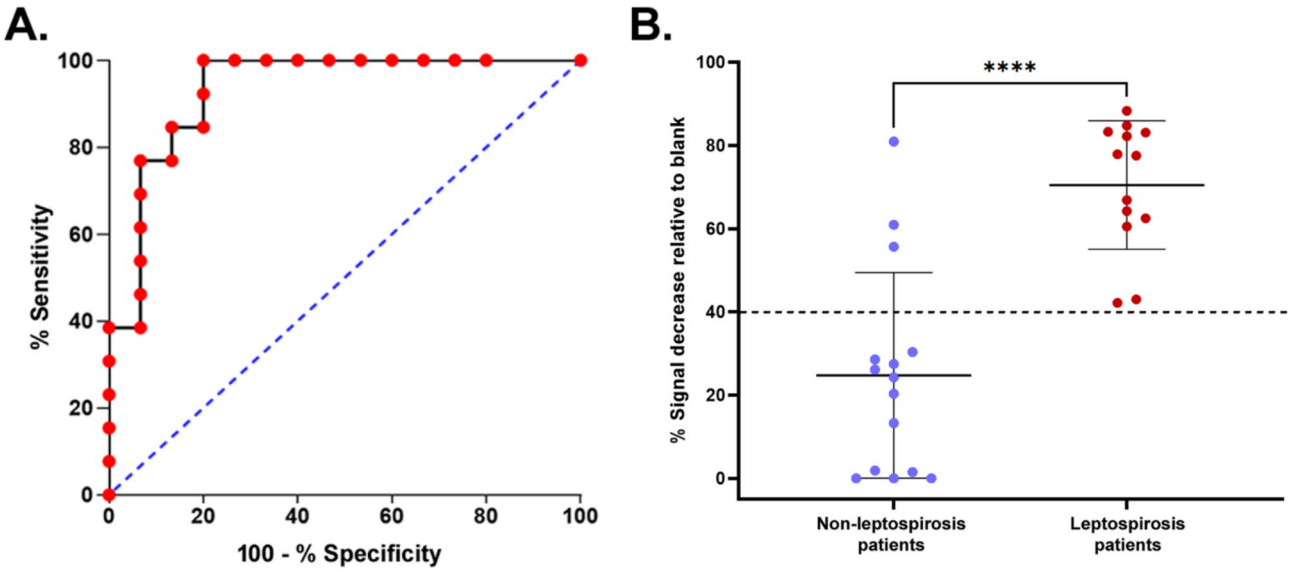

Fig. 2. Receiver operating characteristic (ROC) curve analysis for estimation of cut-off value showing the diagnostic performance of the aptasensor with an area under the curve (AUC). (**A**): Overall, the ROC curve for the diagnostic performance with an AUC of 0.93. (**B**): A scatter plot showing the percentage decrease in signal relative to the blank (mean ± standard deviation) for patients with leptospirosis and those without. The mean percentage signal decrease is significantly higher in leptospirosis patients compared to non-leptospirosis patients ($p < 0.0001$, Mann–Whitney U test). The dashed line indicates an optimal cut-off value at the 40% signal decrease relative to the blank.

Cut-off:	Sensitivity %	Specificity %	AUC
% Signal decrease	(95% CI)	(95% CI)	(95% CI)
30%	100.0	73.3	0.87
	(75.3–100.0)	(44.9–92.2)	(0.72–1.00)
40%	**100.0**	**80.0**	**0.90**
	(75.3–100.0)	**(51.9–95.7)**	**(0.77–1.00)**
50%	84.6	80.0	0.82
	(54.6–98.1)	(51.9–95.7)	(0.66–0.99)
60%	84.6	86.7	0.86
	(54.6–98.1)	(59.5–98.3)	(0.70–1.00)
70%	53.9	93.3	0.74
	(25.1–80.8)	(68.1–99.8)	(0.54–0.93)

Table 1. Analytical values with the area under the curve (AUC) of the percentage decrease in signal relative to the blank.

for reliably distinguishing leptospirosis cases with high diagnostic accuracy (Fig. 2B). At this threshold, the diagnostic performance of the assay achieved a sensitivity of 100%, and a specificity of 80.0% (Table 1).

Comparison of the percentage decrease in signal relative to the blank between leptospirosis and non-leptospirosis groups

The percentage decrease in signal relative to the blank for 13 leptospirosis and 15 non-leptospirosis patients showed a significant difference between the two groups (Fig. 2B). Leptospirosis cases exhibited a higher median (77.6% vs. 24.3%) and interquartile range (IQR) of signal decrease [(61.5, 83.2) vs. (1.5, 30.3)] compared to non-leptospirosis cases, with similar trends observed in the mean ± standard deviation (70.49 ± 15.43 vs. 24.77 ± 24.68). Statistical analysis using the Mann-Whitney U test confirmed this difference as highly significant ($p < 0.0001$). These findings highlight the diagnostic potential of the aptasensor assay, particularly with an optimal cut-off value of 40% signal decrease.

Concordance of aptasensor and PCR assays with the reference MAT

The diagnostic agreement of the aptasensor assay (using a cut-off value at 40% of signal decrease) and the PCR method were evaluated against leptospirosis patients identified by the laboratory-confirmed MAT. This

	Leptospirosis status					
		Case	Control	*Total*		
Aptasensor	Positive	13	3	16	% Positive agreement (Low – High limits)	100.0 (77.2–100.0)
	Negative	0	12	12	% Negative agreement (Low – High limits)	80.0 (54.8–93.0)
	Total	13	15	28	% Overall agreement (Low – High limits)	89.3 (72.8–96.3)
					Kappa (95% CI)	0.79 (0.57–1.00)
PCR	Positive	7	0	7	% Positive agreement (Low – High limits)	53.8 (29.1–76.8)
	Negative	6	15	21	% Negative agreement (Low – High limits)	100.0 (79.6–100.0)
	Total	13	15	28	% Overall agreement (Low – High limits)	78.6 (60.5–89.8)
					Kappa (95% CI)	0.56 (0.27–0.84)

Table 2. Concordance of the aptasensor and PCR diagnostic tests with leptospirosis status by the MAT.

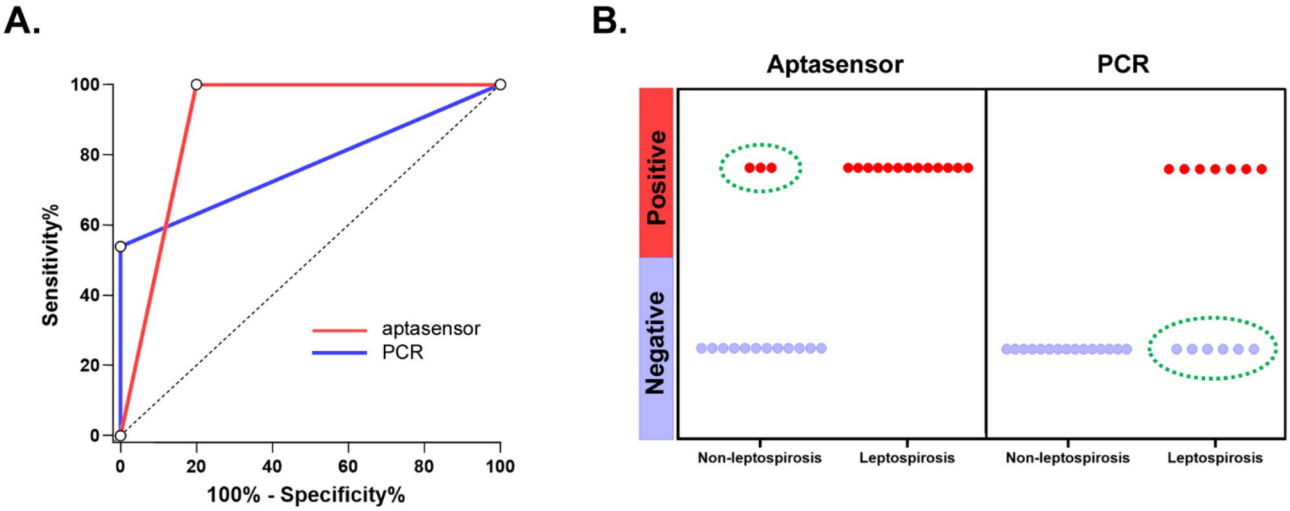

Fig. 3. The diagnostic agreement between the aptasensor using optimum cut-off at 40% signal decrease threshold and PCR methods among leptospirosis and non-leptospirosis patients. (**A**): ROC curves exhibit sensitivity and specificity for the aptasensor (red) and PCR (blue) in identifying leptospirosis. (**B**): Scatter plots show the classification of leptospirosis and non-leptospirosis patients based on the aptasensor and PCR results. Positive and negative classifications are represented by red and purple dots, respectively. The green dotted circles indicate the discordant results.

	PCR					
		Positive	Negative	Total		
Aptasensor	Positive	7	9	7	% Positive agreement (Low – High limits)	100.0 (64.6–100.0)
	Negative	0	12	21	% Negative agreement (Low – High limits)	57.1 (36.5–75.5)
	Total	7	21	28	% Overall agreement (Low – High limits)	67.9 (49.3–82.1)
					Kappa (95% CI)	0.40 (0.14–0.66)

Table 3. Concordance between the aptasensor and PCR tests.

evaluation was based on the first plasma samples collected from leptospirosis patients (cases) as positive and non-leptospirosis patients (controls) as negative, as shown in Table 2. Compared to MAT, the aptasensor demonstrated a higher percentage agreement than PCR (89.3% vs. 78.6%). Kappa analysis revealed that the aptasensor exhibited substantial agreement with kappa value of 0.79, while the PCR method showed moderate agreement with kappa value of 0.56.

Concordance measurement between aptasensor and PCR

The diagnostic performance of the aptasensor and PCR methods in distinguishing patients with and without leptospirosis is presented in Fig. 3A. The ROC curves indicate that the aptasensor and PCR methods achieved AUC values of 0.90 (95% CI: 0.77–1.00, $p < 0.001$) and 0.77 (95% CI: 0.58–0.96, $p < 0.05$), respectively. The concordance rates between the aptasensor and PCR assays are detailed in Table 3. The two methods revealed a

concordance rate of 67.9%, with a fair agreement (Kappa = 0.40). Notably, the positive agreement in the PCR-positive group was 100%, higher than the negative agreement of 57.1% in the PCR-negative group.

Discordant results, where the aptasensor was positive but PCR was negative, were observed in 9 out of 28 samples (32.2%) (Fig. 3B). To investigate these discordant results, comparisons were made with MAT results from the first samples, as shown in Supplementary Table 1. Among the discordant samples, six (46.2%) were from the leptospirosis group and exhibited high MAT titers (800–12,800), while three (20%) were from the non-leptospirosis group and had negative MAT titers (< 50–100).

Patterns of leptospiral infections in endemic areas

Leptospirosis is an endemic disease globally, including in Thailand, where recurrent infections caused by pathogenic Leptospira are common. This study identified three patterns of leptospiral infection: seroconversion (S), indicating an initial infection; high titer (H), representing recurrent infections with the same or closely related serogroups; and seroconversion combined with high titer (S + H), reflecting both initial and recurrent infections. Among the 13 leptospirosis patients studied, 8 (61.5%) exhibited evidence of multiple infections, with the Shermani serogroup being the most frequently associated with prior infections. The aptasensor assay detected all (100%) of the cases confirmed by MAT, demonstrating superior sensitivity compared to PCR (53.8%) (Supplementary Table 1).

Discussion

Leptospirosis is predominantly characterized by subclinical or mild, self-limiting systemic symptoms; however, recent years have seen an increase in severe cases of the disease[1,3,4]. Early and accurate diagnosis is critical to mitigating the high mortality rates associated with severe forms of leptospirosis. Laboratory diagnostic testing for leptospirosis includes serological assays, culture methods, and molecular techniques such as PCR[2,6,7]. The development of rapid and precise diagnostic tools, particularly for the acute phase, is imperative to prevent the progression from mild disease to severe, life-threatening infection. Deaths and serious consequences can be avoided by starting doxycycline treatment as soon as symptoms appear[16].

According to our previous findings, the aptasensor, a particular aptamer for the electron transfer flavoprotein beta subunit (EtfB), demonstrated the potential of a chemical antibody to identify EtfB proteins as antigens for leptospirosis diagnosis[8]. Excellent outcome measures using the aptasensor demonstrated the limits of detection (LOD) for the 6xHis-EtfB protein at 20 nM (0.6 ug/mL). Nevertheless, the aptasensor assay has not yet been tested on clinical samples. The aim of this investigation was to assess the accuracy of the aptasensor as an antigen test in clinical samples during an acute stage of leptospirosis. To identify the optimal cut-off value for the electrochemical biosensor results, this is the first study to incorporate the percentage signal decrease calculated relative to the blank. Several laboratory-developed diagnostic tests produced good results, but their outcomes in actual clinical samples were controversial. In this study, the optimal cutoff point for evaluating the diagnostic accuracy of the aptasensor assay, with an AUC of 0.90, was determined to be a 40% signal decrease relative to the blank. This threshold effectively distinguished between leptospirosis and non-leptospirosis groups, ensuring high diagnostic accuracy. A comparative analysis of the aptasensor assay and MAT demonstrated superior sensitivity in early diagnostic capabilities for the aptasensor assay. The MAT, while a traditional reference method for leptospirosis diagnosis, is hindered by its dependence on seroconversion and the requirement for paired serum samples from a patient with suspected leptospirosis to demonstrate MAT with rising titers, which delays diagnosis during the critical acute-phase serum samples 5–7 days after illness[17,18].

To our knowledge, this study represents the first to report the aptamer-based assay as an antigen test in actual clinical samples. The developed aptasensor assay performed only on the first visit samples revealed a sensitivity of 100% and a specificity of 80.0%. The findings showed that leptospirosis can be diagnosed early with this extremely potent assay. Only a few publications utilizing monoclonal antibody (MAb) for leptospirosis diagnosis were created to improve the antigen tests. The findings are consistent with the two reports that the MAb testing detected leptospiral antigens. In these studies, the sensitivity and specificity of an LFA containing 1H6 monoclonal antibody for identifying *Leptospira* lipopolysaccharide antigen in patient and animal urine samples were 89% and 87%, respectively[19]. In another study, the *Leptospira* antigenuria tested by the MAb-based dot-ELISA using MAb LD5, which reacted to the 35- to 36-kDa components in urine samples on days 1, 2, 3, 7, and 14 of hospitalization, yielded positive results for 75.0, 88.9, 97.2, 97.2, and 100%, respectively[20]. To find leptospiral antigens in the plasma samples from patients with fever, MAb-based ELISA was created[21]. Our study also aligns with previous reports employing MAb-based assays for antigen detection of leptospirosis. However, unlike prior reports that predominantly focused on urine samples, this study highlights the utility of acute plasma derived from whole blood as a diagnostic specimen. Leptospirosis is disseminated bacteremic infection; the leptospires are first present in the blood in the first few days after exposure and during the acute stage of illness, which lasts from 3 to 10 days before disseminating to the target organs of the liver and kidney[2,7]. Urine leptospires start to appear in the second week of clinical disease and can last anywhere from one to several weeks[22]. Blood-based antigen detection facilitates early diagnosis during the bacteremic phase, which precedes renal colonization and urinary shedding. While antigen detection in urine can be diagnostic, it may reflect active infection or colonization, making early blood-based diagnostics more relevant for timely clinical intervention[23]. Although the window for detecting organisms in the bloodstream is smaller, up to roughly a week after the onset of clinical disease, acute whole blood is the best specimen type for a timely diagnosis[24].

Since PCR using blood samples was a preferable method recognized for the early diagnosis of leptospirosis[25–30], the concordance rate for the aptasensor was compared with the reference PCR. Even in situations where the MAT results were negative (MAT titer < 50 to 100), all 5 (100%) initial samples taken within 7 days of the onset of illness from leptospirosis cases demonstrating seroconversion gave positive results for the PCR and aptasensor assays in this study, greatly enhancing the capacity for early diagnosis. The sensitivity of MAT is comparatively

low in the first acute-phase samples (30%), but it rises to 63% in the second acute-phase samples and 76% in the convalescent-phase samples, according to a previous study[22]. According to these results, PCR and aptasensor provide accurate diagnostic testing in the early stages of infection. A comparison between aptasensor and PCR demonstrated that the concordance rate revealed 67.9% with a fair agreement. The positive agreement (100%) in the PCR-positive group was higher than the negative agreement (57.1%) in the PCR-negative group. Notably, in leptospirosis patients with high MAT titers (800–12,800), the aptasensor assay yielded positive results in 100% of cases, whereas PCR detected the leptospiral DNA in only 25% of cases. This discrepancy could be attributed to rapid antibody-mediated clearance of leptospires from the bloodstream[2,7] or the possibility that abundant antigen production from mRNA translation in the presence of rRNA persists despite DNA clearance. These findings indicated the aptasensor assay as a powerful diagnostic tool, particularly for detecting antigens in initial and recurrent infections, regardless of disease prevalence.

There are various limitations to our study. To obtain meaningful results, the number of clinical samples should be increased. Additionally, the clinical samples may be gathered from a limited geographic area rather than the entire country. Another point is the absence of leptospiral antigen kinetics at the time of infection series blood collection.

Methods

Ethical consideration

Ethical approval was obtained from the Human Research Ethics Committee of Thammasat University, Thailand (COE No. 009/2564) and the Institutional Ethics Committee of the Faculty of Medicine, Chulalongkorn University (IRB no. 675/63). Informed consent was obtained from all participants, and their anonymous plasma samples were decoded to unidentified persons in the study. All methods were performed under the relevant guidelines and regulations.

Source of clinical samples

The study was conducted with human plasma samples from 28 patients in Thailand, including 13 patients with confirmed leptospirosis by MAT and 15 negative patients with non-leptospirosis. To facilitate the early-phase diagnosis of leptospirosis, the aptasensor assay was performed using the first acute-phase plasma samples collected within seven days of symptom onset. Confirmatory diagnostic testing was performed using the MAT as the reference standard diagnostic method recommended by the World Health Organization (WHO)[16]. A diagnosis of leptospirosis in paired-serum samples was established based on any of the following MAT criteria: (i) seroconversion, (ii) a four-fold rise in antibody titers between acute and convalescent samples, or (iii) a single acute titer of ≥ 1:400 with a positive PCR result. The MAT employed a panel of 24 leptospiral serogroups/serovars of *L. interrogans* for serological analysis, i.e., Australis, Autumnalis, Ballum, Bataviae, Canicola, Cellidoni, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis strain Hebdomadis, Icterohaemorrhagiae strain RGA, Javanica, Louisiana, Manhao, Mini, Panama, Pomona, Pyrogenes strain Salinem, Ranarum, Sarmin, Sejroe, Shermani, Tarassovi, and Semaranga. All 28 patients had paired-serum samples except two patients (#L06 and #L07) diagnosed with leptospirosis based on a positive MAT titer (≥ 1:400) and positive PCR results in the first samples due to the unavailability of the second samples.

The sample preparation for PCR was performed by collecting EDTA whole blood and whole blood without anticoagulants from patients suspected of leptospirosis. The serum was separated from the whole blood after coagulation by centrifugation and was subsequently used for MAT. PCR was performed directly on EDTA whole blood samples. Serum and plasma samples were stored at -80 °C. The aptasensor assay was performed using leftover plasma samples. All 28 plasma samples with MAT and PCR results were provided by the Faculty of Medicine, Chulalongkorn University.

Evaluation of the aptasensor assay

In alignment to diagnose leptospirosis during its early phase, the diagnostic performance and concordance of the aptasensor assay were evaluated using acute-phase plasma samples collected within seven days of symptom onset.

To assess diagnostic performance, the aptasensor assay results were compared against the leptospirosis status comprising 13 leptospirosis cases and 15 non-leptospirosis controls, identified by reference standard MAT. Sensitivity and specificity were determined using receiver operating characteristic (ROC) analysis.

For test concordance, the aptasensor assay results were compared with those of the reference quantitative PCR (qPCR) method, performed on EDTA-treated whole blood samples from the same group ($n = 28$), including seven PCR-positive and 21 PCR-negative cases. The qPCR assay targeted the *lipL32* gene, a specific marker of pathogenic *Leptospira* species, with a previously established detection limit of 10^2 cells/mL, as determined using spiked blood samples containing *Leptospira interrogans* serovar Pomona[15].

Electrochemical aptasensor assay of EtfB

The electrochemical aptasensor assay was performed as previously described, with some modifications[8]. The principle of the assay and the assay procedures are shown in Fig.4A, B, respectively. First, the deoxyoligonucleotide probe with the sequence complementary to the EtfB aptamer, i.e., ETFB3-63AS, was immobilized onto the streptavidin magnetic beads. Briefly, 100 µL (1 mg) of streptavidin-coupled magnetics beads (Dynabeads™ MyOne™ Streptavidin T1, Thermo Fisher Scientific, USA) were washed three times with PBST (20 mM PBS pH 7.4 containing 0.05% tween) and reconstituted in 184 µL of PBS. Then, 4 µL of 100 µM biotinylated ETFB3-63AS antisense probe (5'-biotin-TEG-ATTCTACAAGGTCAGTAGGAAT 3') were added to the resuspended streptavidin beads and the remaining biotin-binding sites of the beads were subsequently blocked by adding 12 µL of 100 µM biotinylated poly-dT (5'-biotin-TEG-TTTTTTTTTT 3'); both oligos were obtained from

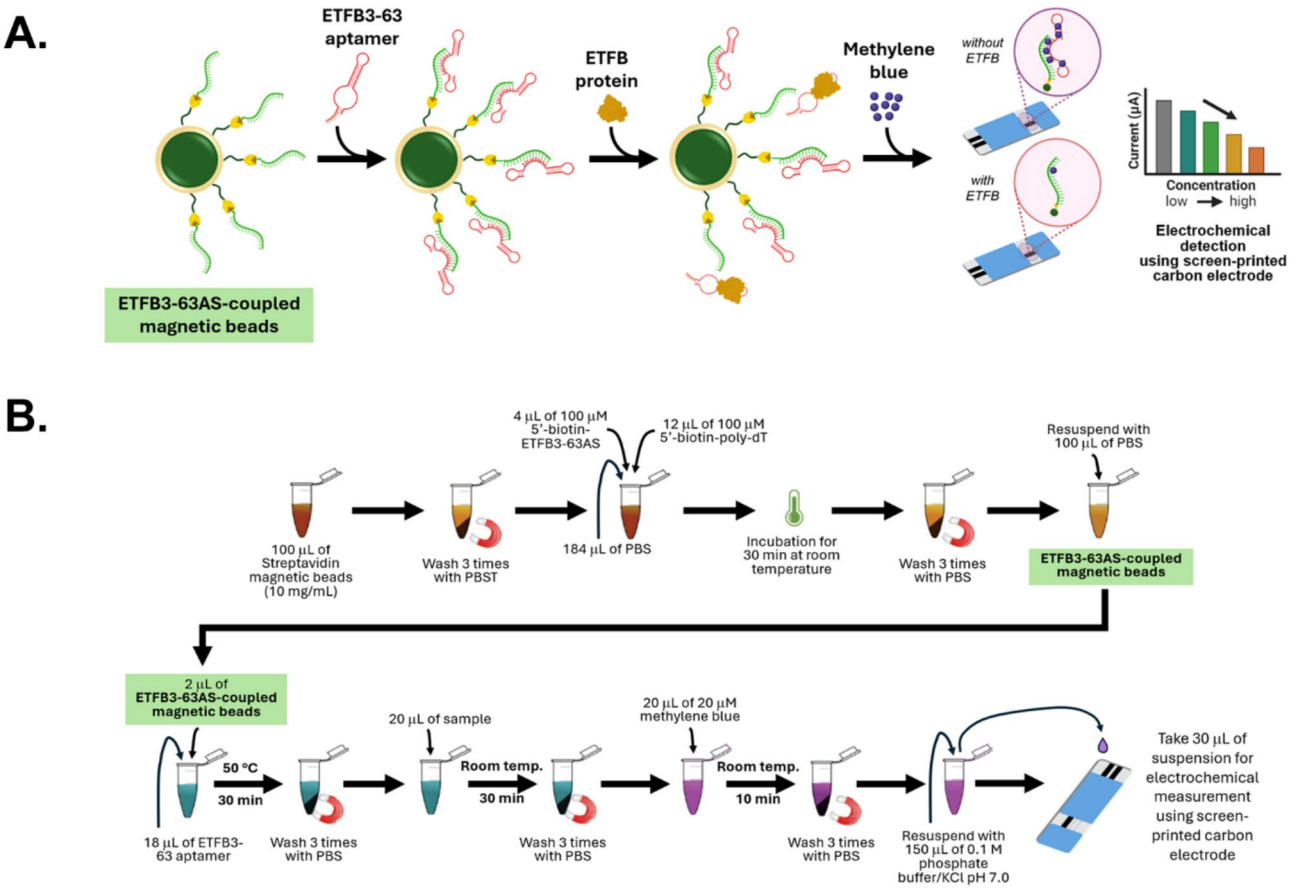

Fig. 4. Schematic diagrams of the electrochemical aptasensor for detection of the EtfB protein of *Leptospira interrogans*. (**A**): The principle of the assay and (**B**): The assay procedures.

Macrogen, Republic of Korea. This reaction was incubated for 30 min at room temperature with continuous rotation. After incubation, the ETFB3-63AS-coupled streptavidin magnetic beads were washed and resuspended in 100 µL of PBS.

For each assay, 2 µL of the ETFB3-63AS-coupled bead suspension above were mixed with 18 µL of 100 µM the ETFB3-63 aptamer (5' aaaaaaaa 3'); the aptamer was transcribed in vitro as previously described with the pyrimidine nucleotides substituted with 2′-fluoro-deoxycytidine and 2′-fluoro-deoxyuridine[8] and the underlined sequence anneals to the ETFB3-63AS antisense probe. The reaction was incubated at 50 °C for 30 min to enable hybridization of the aptamer to the antisense probe. Following hybridization, the beads were washed three times with PBS. The hybridized beads were incubated with 20 µL of the sample for 30 min at room temperature followed by three washes with PBS. Subsequently, 20 µL of 20 µM methylene blue solution was added to the beads, and the reaction was incubated for 10 min at room temperature. The beads were washed three times with PBS and resuspended in 150 µL of 0.1 M KCl in PBS. A 30-µL aliquot of the bead suspension was applied to a screen-printed carbon electrode, and differential pulse voltammetry (DPV) signals were measured using a PalmSens4 potentiostat with PSTrace software version 5.9 (PalmSens BV, Utrecht, the Netherlands). The DPV experimental parameters were as follows: a scanning range (−0.5 to −0.1 V), scan rate of 0.1 V/s, step potential of 0.01 V, modulation amplitude of 0.05 V, and interval of 0.05 s. The DPV analysis was measured three times for each sample. To quantify the EtfB concentration based on the DPV signals, the peak current intensity of the sample (I_{Sample}) and the blank (I_{Blank}) were measured and used to calculate the percentage decrease in signal relative to the blank, i.e., $(I_{Blank} - I_{Sample}) \times 100/I_{Blank}$; the negative values were treated as 0%.

Statistical analysis

Data analysis was performed using SPSS software (version 29). The comparative performance of the aptasensor assay and PCR, with MAT as the reference standard, was evaluated using the chi-square test. Diagnostic performance metrics, including sensitivity and specificity, were calculated to assess the effectiveness of the aptasensor assay. Percent signal decreases relative to the blank were reported as the median and interquartile range (IQR) and analyzed using the Mann-Whitney test. Cut-off values were determined using receiver operating characteristic (ROC) curve analysis[32]. The discriminative performance of the test was assessed by the area under the curve (AUC), with higher AUC values indicating higher predictive capability. The AUC, along with 95% confidence intervals (CI), was used to compare the diagnostic performance.

Percent agreement and Cohen's kappa values were calculated using GraphPad's online tool (available at https://www.graphpad.com/quickcalcs/kappa1). The interpretation of Cohen's kappa values followed standard guidelines: kappa < 0 indicates no agreement; 0.01–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; and 0.81–1.00, almost perfect or perfect agreement. A p-value of less than 0.05 was considered statistically significant for all analyses.

Data availability

All data analyzed during this study are included in this published article and its supplementary information file.

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Author contributions
U.K. contributed to the study's conception and design. C.S., B.L., and K.P. analyzed the data. C.T., S.S., T.C., and C.P. performed the experiments. C.K. and W.W. contributed to the provision of samples. U.K. and C.S. wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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