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L. Li[1, 2], H. Liang[1, 2], S. Zhong[1, 2], C. Chen[2],
V. N. Leont'yev[1], I. V. Voitau[1]
[1]Belarusian State Technological University
[2]Guangzhou Youdi Bio-technology Co., Ltd. (People's Republic of China)

ESTABLISHMENT OF TIME-RESOLVED FLUORESCENCE IMMUNOCHROMATOGRAPHIC METHOD FOR *TOXOPLASMA GONDII* DETECTION

Toxoplasma gondii (*T. gondii*) is a parasitic zoonosis that causes abortion or congenital diseases. The purpose of this study is to establish a immunochromatographic assay (ICA) method using time-resolved fluorescence microspheres (TRFM) for *T. gondii* quantitative detection in serum.

Firstly, we activated the TRFM and then coupled with anti-*T. gondii* antibodies (mAb1) and anti-BSA-DNP antibodies to prepare the TRFM-mAb1 probes and TRFM-BSA-DNP probes. Then, we optimized the coupling pH, T line antibody concentration, probes usage and detection time. Finally, we established the *T. gondii*-TRFMICA method and assemble the *T. gondii*-TRFMICA kit, the standard curve, sensitivity, precision, specificity, clinical sensitivity and specificity were evaluated.

Under the optimized conditions, the *T. gondii*-TRFMICA test was achieved within 20 min with the sensitivity 0.1 ng/mL. The recoveries were ranging from 100–110% with the intra-assay and inter-assay *CV* lower than 10%. The kits have detection specificity for positive serum of several common infectious diseases and some common serum components, and have high detection sensitivity and specificity in human and cat clinical samples.

A TRFMICA kit for *T. gondii* quantitative detection was successfully prepared with high sensitivity, specificity, precision and clinical sensitivity and specificity. We recommend TRFMICA as a promising technique in the clinical diagnosis and monitoring of toxoplasmosis in human and animals.

Keywords: *Toxoplasma gondii*, time-resolved fluorescence microsphere, immunochromatographic assay, parasite, zoonosis.

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Л. Ли[1, 2], Х. Лян[1, 2], Ш. Чжун[1, 2], Ц. Чен[2],
В. Н. Леонтьев[1], И. В. Войтов[1]
[1]Белорусский государственный технологический университет
[2]Компания Guangzhou Youdi Bio-technology Co., Ltd. (Китайская Народная Республика)

РАЗРАБОТКА ФЛУОРЕСЦЕНТНОГО ИММУНОХРОМАТОГРАФИЧЕСКОГО МЕТОДА С ВРЕМЕННЫМ РАЗРЕШЕНИЕМ ДЛЯ ОБНАРУЖЕНИЯ *TOXOPLASMA GONDII*

Toxoplasma gondii (*T. gondii*) – паразитарный зооноз, вызывающий выкидыши или некоторые врожденные заболевания. Целью данного исследования является разработка метода иммунохроматографического анализа (ICA) с использованием флуоресцентных микросфер с временным разрешением (TRFM) для количественного обнаружения *T. gondii* в сыворотке крови.

Для исследования сначала активировали TRFM, а затем связали с антителами против *T. gondii* (mAb1) и антителами против BSA-DNP для приготовления зондов TRFM-mAb1 и зондов TRFM-BSA-DNP.

После оптимизировали pH, концентрацию антител Т-линии, использование зондов и время обнаружения. Наконец разработали метод *T. gondii*-TRFMICA и сформировали набор *T. gondii*-TRFMICA, оценили линейность, чувствительность, прецизионность, специфичность, а также клинические чувствительность и специфичность.

При оптимальных условиях тест *T. gondii*-TRFMICA выполнялся в течение 20 мин с чувствительностью 0,1 нг/мл. Открываемость составляла от 100 до 110%, при этом внутрилабораторный и межлабораторный коэффициент вариации был ниже 10%. Наборы обладают специфичностью обнаружения нескольких распространенных инфекционных заболеваний в клинических образцах человека и кошек.

Набор TRFMICA для количественного обнаружения *T. gondii* успешно создан и обладает высокой чувствительностью, специфичностью, прецизионностью, а также клиническими чувствительностью и специфичностью. Набор TRFMICA рекомендуем как перспективный метод клинической диагностики и мониторинга токсоплазмоза у человека и животных.

Ключевые слова: *Toxoplasma gondii*, флуоресцентная микросфера с временным разрешением, иммунохроматографический анализ, паразит, зооноз.

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Introduction. *Toxoplasma gondii* (*T. gondii*) is an obligate intracellular parasite that causes toxoplasmosis. As a parasitic zoonosis, it can cause abortion or congenital diseases in immunocompromised individuals and animals [1, 2]. Molecular, serology and bioassay analysis had confirmed the high prevalence of *T. gondii* infection in domestic or wild animals and human, and there is an important risk of human infection in consuming raw or undercooked sheep/lamb meat, unpasteurized milk and dairy products, and contacting with domestic or wild feline feces [3, 4]. Toxoplasmosis constitutes a challenge for public health, animal production and welfare. However, only a limited drugs for *T. gondii* has been marketed for clinical applications [3]. Accurate screening and early diagnosis are keys to *T. gondii* controlling.

Up till now, molecular testing is the most accurate and commonly adopted method, which requires sophisticated equipment with time-consuming pretreatment in laboratory [5]. In addition to classical screening, the establishment of more new test methods may become practical and convenient. Considering that the strip method is simple and convenient for users, time-resolved fluorescence immunochromatographic assay (TRFICA) has been widely used owing to higher sensitivity and stability [6, 7]. In this study, a Europium (Eu) nanosphere-based TRFICA for the rapid screening of *T. gondii* was developed, and it provides a favorable tool for clinical diagnosis and monitoring of toxoplasmosis in human and animals.

Main part. Toxoplasma lysate antigens (TLA) (mouse ascites) and its monoclonal antibodies pair (mAb1 and mAb2) were prepared in our own laboratory. Time-resolved fluorescence microspheres (TRFM, particle size 200 nm) were purchased from Suzhou Vdo Biotech Co., Ltd. (#FT0200CA, China). Dinitrophenol (DNP)-BSA antigen (DNP-BSA) and its DNP monoclonal antibody came from Seebio. 1-(3-Dimethylaminyl)-3-ethylenediamine hydrochloride (EDC) and N-hydroxysuccinimide (NHS) came from Beyotime and Acmec. Sample pad, bonding pad, nitrocellulose (NC) membrane, absorbent paper and polyvinyl chloride (PVC) soleplate came from AUTOKUN. The healthy control serum and positive serum samples of *T. gondii*, cryptosporidium, schistosome, canine distemper virus (CDV), canine parvovirus (CPV), chlamydia and brucella came from Guangzhou Center for Disease Control and Prevention, and South China Agricultural University.

After classical EDC/NHS activation, the TRFM were coupled with mAb1 and then blocked by BSA to obtain TRFM-mAb1 probes. Briefly, after washing with 10 mM 2-(N-morpholine)-ethanesulfonic acid solution (pH 6.2), 35 μL EDC (10 mg/mL) and 330 μL NHS (10 mg/mL) were added into the TRFM for activation, 37°C, 40 rmp/min, 15–30 min, in dark. After centrifugation, mAb1 was added and subjected to shaking coupling at 37°C for 2 h. After that, the TRFM was blocked in 1% BSA solution for 1 h at 37°C. After centrifugation again, obtained the TRFM-mAb1 probes. The preparation steps of TRFM-BSA-DNP probes is the same as the above method. The prepared TRFM-mAb1 probes and TRFM-BSA-DNP probes were stored at 4°C in dark. The coupling pH was optimized.

The NC membrane has a test line (T line) and a control line (C line), which were respectively coated

by mAb2 and DNP mAb. Coating was performed using the spraying platform at the spraying speed 0.8 μL/cm. Sample pads were soaked in a buffer (12 mmol/L sodium tetraboric acid, 1% polyvinyl pyrrolidone (PVP), 0.3% sodium casein, 1% Trition-X100, 0.05% ProClin 300, pH 7.8) for 2 h, and bonding pads were soaked in another buffer (60 mmol/L $Na_2HPO_412H_2O$, 0.5% PVA, l% Trition-X100, 0.8% BSA, 0.05% ProClin 300, pH 7.4) for 2 h. After coating or soaking, NC membranes, sample pads and bonding pads were dried in a 37°C air drying oven for 2 h, and then sealed and stored in a dry environment. The T line coating concentration of mAb2 was optimized.

The *T. gondii*-TRFMICA kit consist of 2 components: card and probes tube. The sample pad, bonding pad, NC membrane, and absorbent paper are sequentially fixed on the PVC board, and cut into 3 mm wide strip, loaded into a plastic cartridge, to form the *T. gondii*-TRFMICA card. The probes tube contains freeze-dried TRFM-mAb1 probes and TRFM-BSA-DNP probes. The *T. gondii*-TRFMICA kit stores in a dark and dry environment at room temperature.

The reaction mode of *T. gondii*-TRFMICA is the double antibody sandwich method. The whole test procedure is simple and convenient: Take 50 μL sample and some probes, mix well and incubat for 5 min, and then dripp into the sampling slot of the *T. gondii*-TRFMICA card. After that, insert the card into the card slot of the time resolved fluorescence quantitative analyzer, the analyzer will automatically perform the test and print the test report. The concentration of *T. gondii* in sample was automatically obtained using the built-in standard curve. The probes usage and detection time was optimized.

In order to improve the performance of *T. gondii*-TRFMICA, this study optimized the coupling pH (5.5, 6.5, 7.5 and 8.5), T line antibody concentration (1.0, 1.5, 2.0 and 2.5 mg/mL), and probes usage (5.0, 8.0, 10.0 and 12.0 μL). The fluorescence intensity of T line (FT) and C line (FC) was recorded. Fluorescence intensity as the left vertical axis, FT/FC as the right vertical axis, and the parameter to be optimized as the horizontal axis to determine the optimal conditions.

Furthermore, we prepared the strips using the optimal conditions determined above. The 100 ng/mL TLA as the sample to perform the test, recorded the time (6, 9, 12, 15, 18, 21, 24, 27 and 30 min) and its corresponding fluorescence intensity. For determination of detection time a curve was drawn of fluorescence intensity depending on detection time. Three replicates of each optimization.

According to the optimal *T. gondii*-TRFMICA conditions and test procedure, 0, 0.1, 1.0, 10.0, 50.0, 100.0 and 200.0 ng/mL series TLA were used to determine the standard curve and sensitivity of this method, three replicates of each concentration. The concentration values of series TLA were plotted on the X-axis, and the mean values of fluorescence intensity were plotted on the Y-axis, performed the linear fit and drawn the standard curve, and determined the sensitivity.

Dilute TLA to 100.0, 10.0, and 1.0 ng/mL using normal human serum, and their fluorescence values were respectively measured by the one and three batches of kits to calculate the coefficient of variation (*CV*) and recovery. Three replicates of each concentration. Recovery (%) = = (determined concentration – basal concentration) / spiked concentration × 100%. *CV*(%) = = SD / mean × 100%.

The positive serum samples of *T. gondii*, cryptosporidium, schistosome, CDV, CPV, chlamydia and brucella, and the common components in serum (C-reactive protein (hs-CRP), interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), hemoglobin, bilirubin, and cholesterol) were selected as sample to be tested by the *T. gondii*-TRFMICA kits for the specificity assay.

8 clinical positive 15 clinical negative serum samples of human, 5 positive and 10 negative serum samples of cats were used for clinical samples evaluation. All serum samples were diluted 100-fold with 0.01 mol/L PBS, and then were tested by *T. gondii*-TRFMICA kits according to the "Test procedure". Finally, recorded the fluorescence intensity and compared the differences in positive and negative samples.

Data were statistically analyzed and graphed using GraphPad Prism 5 (GraphPad Software, USA). All results are presented as the mean or mean ±*SD*.

The optimal results of coupling pH, T line antibody (mAb2) concentration, probes usage and detection time were shown in Fig. 1.

As the coupling pH increased from 5.5 to 6.5, FT/FC increased. At pH = 6.5, FT/FC value reached the maximum, indicating that pH = 6.5 is the optimal coupling pH. For mAb2 T line concentration, as the concentration increased from 1.0 to 1.5 mg/mL, FT/FC increased and reached the maximum, which indicates 1.5 mg/mL is the optimal concentration of mAb2. 8.0 μL is the optimal probes usage for *T. gondii*-TRFMICA detection. As the detection time gradually increased, FT/FC increased. When the time increased to 15 min, the FT/FC value reached the maximum, and the FT/FC value remained almost unchanged after 15 min. Overall, the optimal conditions for *T. gondii*-TRFMICA method: coupling pH 6.5, T line antibody (mAb2) concentration 1.5 mg/mL, probes volume 8.0 μL and detection time 15 min.

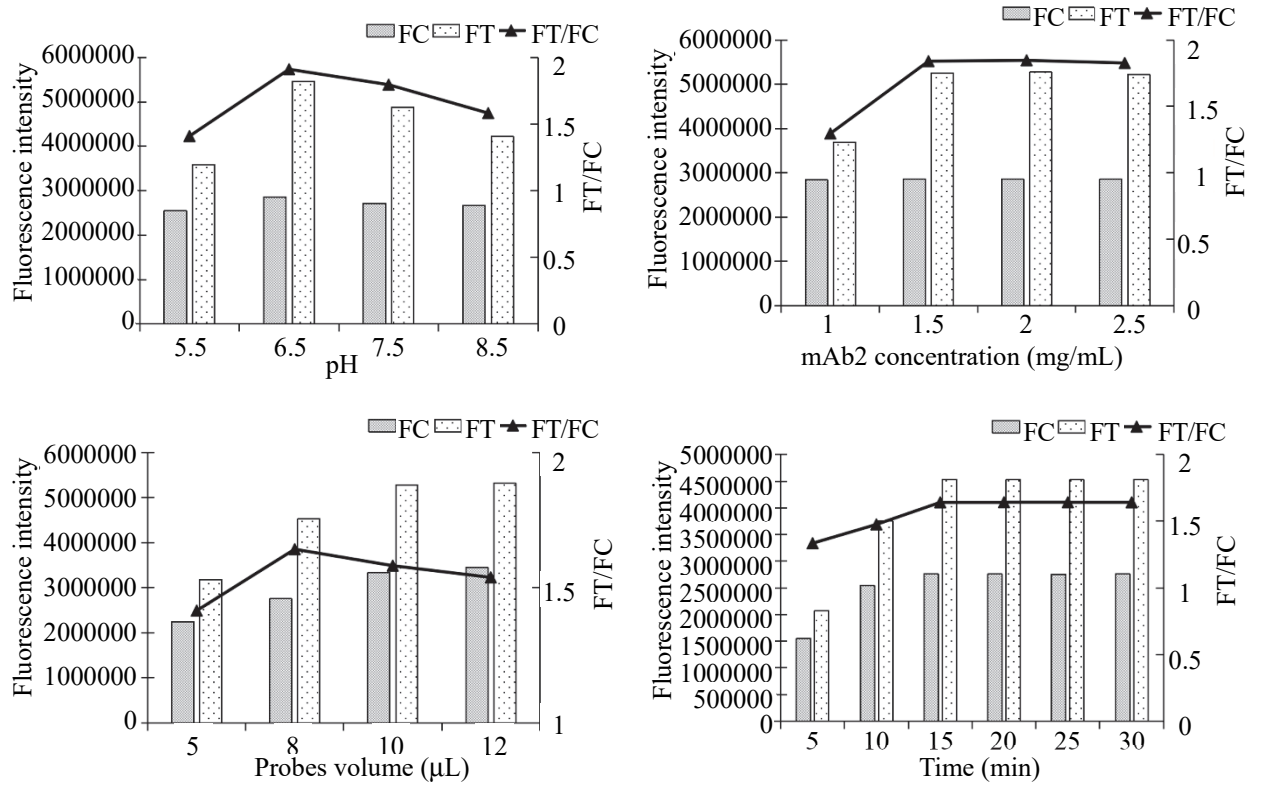

Fig. 1. Optimization conditions of *T. gondii*-TRFMICA method

The standard curve of *T. gondii*-TRFMICA kits is presented in Fig. 2. Curves equation is:

$$y = 0.0138x + 0.1887 \ (R^2 = 0.971),$$

the curve exhibit a well-defined linear relationships in a wide concentration ranges (0–200 ng/mL). The sensitivity by naked eyes was 0.1 ng/mL.

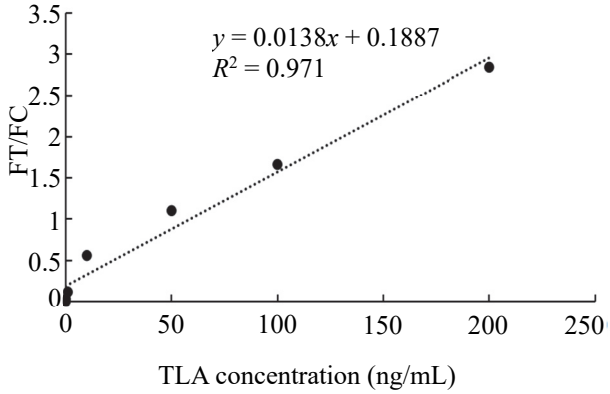

Fig. 2. The standard curve of the *T. gondii*-TRFMICA kits

As shown in Table 1, in intra-array testing, the *CV* was ranging from 1.54 to 4.04%, and the recovery ranged from 103.83 to 108.00%. In inter-array testing, the *CV* was ranging from 4.74 to 6.71%, and the recovery ranged from 105.67 to 108.40%. All *CV*s of the intra- and inter-array in three TLA concentration levels were below 10%, and recovery was between 100–110%. The precision of *T. gondii*-TRFMICA kits was high enough and meet the requirements of clinical immunoassays.

Precision results

	TLA concentration (ng/mL)	mean ± SD	Recovery (%)	*CV* (%)
Intra-array (n=3)	100	103.83 ± 1.59	103.83	1.54
	10	10.80 ± 0.44	108.00	4.04
	1	1.07 ± 0.030	107.00	2.80
Inter-array (n=3)	100	108.40 ± 5.52	108.40	5.09
	10	10.83 ± 0.51	108.33	4.74
	1	1.06 ± 0.071	105.67	6.71

As shown in Fig. 3, for the three dilutions of cryptosporidium, CDV, CPV, chlamydia and brucella samples (1:10, 1:100 and 1:200 dilution), the test results were consistent with those of the 0 ng/mL blank samples.

For the three concentrations of hs-CRP, IL-6, TNF-α, hemoglobin, bilirubin, and cholesterol samples (500, 100 and 10 ng/mL), the test results were also consistent with those of the 0 ng/mL blank samples. Meanwhile, the test results of 0 ng/mL samples were significantly lower than those of *T. gondii* positive serum samples. Therefore, this *T. gondii*-TRFMICA kit is a specific detection method for *T. gondii*.

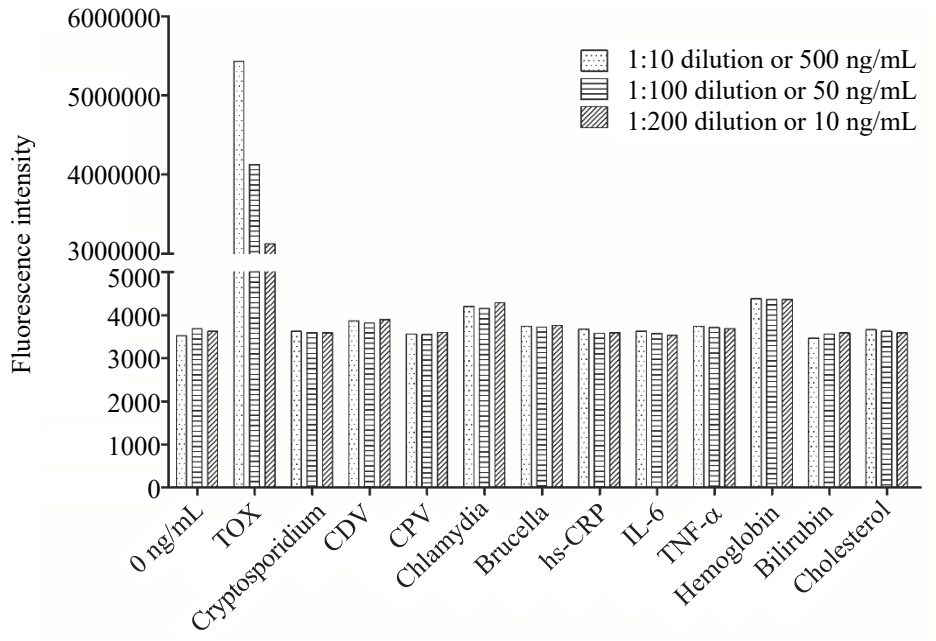

Fig. 3. Specificity results of the *T. gondii*-TRFMICA kits

Serum samples were collected to evaluate the clinical application of *T. gondii*-TRFMICA kits in human and animals. The results showed that the fluorescence intensity of positive samples were significantly higher than that of negative samples (Fig. 4, *a*), and there was a significant statistical difference between positive and negative samples (Fig. 4, *b*), indicating that the *T. gondii*-TRFMICA kits had high detection sensitivity and specificity in human and cat.

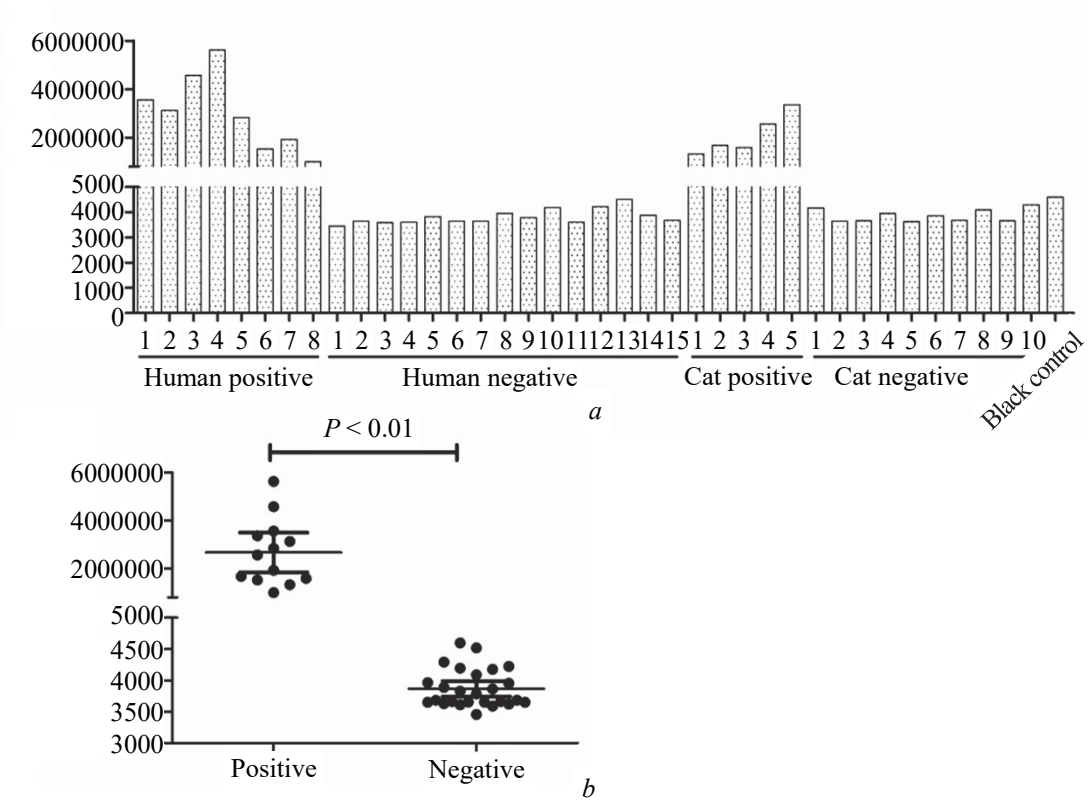

Fig. 4. Testing results of clinical samples:
a – fluorescence intensity of positive samples (human 8, cat 5) and negative samples (human 15, cat 10);
b – statistical comparison of fluorescence intensity between positive and negative samples

Toxoplasmosis is a globally parasitic protozoan disease. According to estimates, one in three people have been exposed to toxoplasmosis, and the incidence is likely substantially underestimated worldwide due to the incomplete screening [8, 9]. Although the *T. gondii* infection is generally asymptomatic for most adults, but it may induce severe complications in women with early pregnancy [10]. Pregnant women with toxoplasmosis has the risk of miscarriage, early delivery, stunted growth, or congenital anomalies [11, 12]. In countries with developed animal husbandry, *T. gondii* infection is more severe. A clinical study from Turkey pointed out that the prevalence of toxoplasmosis in pregnant women was 46.2%, the rate of acute toxoplasma infection was 4%, and the total mother-to-child transmission rate was 5% (5/101) [13]. At present, toxoplasmosis can be diagnosed by various methods, including antibody detection using serological testing and DNA determination by PCR [14]. Serological testing (mostly ELISA antibody testing) is considered the most practical method for diagnosing *T. gondii* [15], but the false-positive or false-negative results often trouble testers [16, 17].

Currently, new methods for diagnosing *T. gondii* are constantly being developed. Aly et al. developed a nano-gold ELISA, that had a significant improvement in diagnosis than the traditional ELISA method (higher specificity and sensitivity) [18]. Considering that the strip methods are simple and convenient, immunochromatographic method has been widely applied in various diseases diagnosis. Further, TRFMICA that combine quantification, sensitivity, and stability have become a more promising approach. Compared with traditional immunochromatographic methods, the performance of TRFMICA has been improved owing to about 10^3 times amplification and detection signal stability [19, 20]. In this study, we attempted to establish a sensitive TRFMICA method for *T. gondii* antigen quantitative detection. Results showed the *T. gondii*-TRFMICA kits has high sensitivity (0.1 ng/mL) and high specificity, the recoveries ranged from 100–110% and the intra-assay and inter-assay *CV* lower than 10%, and have high detection sensitivity and specificity in human and cat clinical samples.

Conclusion. A rapid (20 min), simple, and sensitive TRFMICA method was preliminary established, the prepared *T. gondii*-TRFMICA kits can be adopted for *T. gondii* TLA quantitative detection, with high sensitivity, specificity, precision and clinical sensitivity and specificity. The *T. gondii*-TRFMICA kits prepared in this study is more convenient and fast than the traditional ELISA, and can achieve quantitative detection compared with traditional immunochromatographic methods. This study provides a favorable tool for clinical diagnosis and monitoring of toxoplasmosis in human and animals.

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Information about the authors

Li Laiqing – bachelor, engineer, chairman. Guangzhou Youdi Bio-technology Co., Ltd. (3, Juquan Road, Huangpu District, 510663, Guangzhou, People's Republic of China). E-mail: lilaiqing191@163.com

Liang Huankun – bachelor, assistant engineer, quality manager. Guangzhou Youdi Bio-technology Co., Ltd. (3, Juquan Road, Huangpu District, 510663, Guangzhou, People's Republic of China). E-mail: 605712026@qq.com

Zhong Shuhai – bachelor, assistant engineer, R&D supervisor. Guangzhou Youdi Bio-technology Co., Ltd. (3, Juquan Road, Huangpu District, 510663, Guangzhou, People's Republic of China). E-mail: 1627840617@qq.com

Chen Cuicui – master, engineer, project manager. Guangzhou Youdi Bio-technology Co., Ltd. (3, Juquan Road, Huangpu District, 510663, Guangzhou, People's Republic of China). E-mail: 285020003@qq.com

Leont'yev Viktor Nikolayevich – PhD (Chemistry), Associate Professor, Head of the Department of Biotechnology. Belarusian State Technological University (13a, Sverdlova str., 220006, Minsk, Republic of Belarus). E-mail: leontiev@belstu.by

Voitau Ihar Vital'evich – DSc (Engineering), Professor, Rector. Belarusian State Technological University (13a, Sverdlova str., 220006, Minsk, Republic of Belarus). E-mail: voitov100@mail.ru

Информация об авторах

Ли Лайцин – бакалавр, инженер, председатель. Компания Guangzhou Youdi Bio-technology Co., Ltd. (510663, Гуанчжоу, р-н Хуанпу, ул. Цзюцюань-роуд, 3, Китайская Народная Республика). E-mail: lilaiqing191@163.com

Лян Хуанькунь – бакалавр, помощник инженера, менеджер по качеству. Компания Guangzhou Youdi Bio-technology Co., Ltd. (510663, Гуанчжоу, р-н Хуанпу, ул. Цзюцюань-роуд, 3, Китайская Народная Республика). E-mail: 605712026@qq.com

Чжун Шухай – бакалавр, помощник инженера, руководитель исследований и разработок. Компания Guangzhou Youdi Bio-technology Co., Ltd. (510663, Гуанчжоу, р-н Хуанпу, ул. Цзюцюань-роуд, 3, Китайская Народная Республика). E-mail: 1627840617@qq.com

Чен Цыкуй – мастер, инженер, руководитель проекта. Компания Guangzhou Youdi Bio-technology Co., Ltd. (510663, Гуанчжоу, р-н Хуанпу, ул. Цзюцюань-роуд, 3, Китайская Народная Республика). E-mail: 285020003@qq.com

Леонтьев Виктор Николаевич – кандидат химических наук, доцент, заведующий кафедрой биотехнологии. Белорусский государственный технологический университет (220006, г. Минск, ул. Свердлова, 13а, Республика Беларусь). E-mail: leontiev@belstu.by

Войтов Игорь Витальевич – доктор технических наук, профессор, ректор. Белорусский государственный технологический университет (220006, г. Минск, ул. Свердлова, 13а, Республика Беларусь). E-mail: voitov100@mail.ru

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