A Novel Rapid Visual Detection of *Toxoplasma Gondii* by Combining Recombinase Polymerase Amplification and Lateral Flow Dipstick Coupled With CRISPR-Cas13a Fluorescence Assay

Jinhong Zhao

Wannan Medical College

Yuanyuan Li

Wannan Medical College

Qiqi Xue

Wannan Medical College

Zhiwei Zhu

Wannan Medical College

Minghui Zou

Wannan Medical College

Fang Fang (✉ 30363754@qq.com)

Wannan Medical College

Research

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Abstract

Background: Toxoplasmosis caused by infecting with *Toxoplasma gondii* is a kind of parasitic disease that prevalent all over the world and does great harm to pregnant women and newborns. Effective, rapid and accurate diagnosis *T. gondii* is urgently needed to prevent and treatment the toxoplasmosis. The purpose of this study was to develop a rapid visual detection assay using recombinase aided amplification (RAA) and lateral flow dipstick (LFD) coupled with CRISPR-Cas13a fluorescence, henceforth RAA-Cas13a-LFD, for detection of *T. gondii*.

Methods: Targeting 529bp gene of *T. gondii*, the primers and probes for RAA-Cas13a-LFD assay were designed and screened. The reaction time of RAA-LFD-Cas13a assay was optimized, as well as the sensitivity and specificity was further validated. Finally, the diagnostic performance of *T. gondii* was evaluated using the RAA-Cas13a-LFD assay for clinical blood samples.

Results: The RAA-Cas13a-LFD assay was performed in an incubator block at 37°C within 2h, and the amplicons were visible through LFD for naked eye visualization. The detection limit of the developed RAA-Cas13a-LFD assay was 1×10^{-6} ng/ μ L with high specificity for *T. gondii*. Compared with qPCR assay, there was a consistent positive rate among the clinical blood samples.

Conclusion: In this study, A rapid and visual RAA-Cas13a-LFD assay was developed. It requires no sophisticated equipment and shows promise for on-site surveillance of *T. gondii*.

Background

Toxoplasma gondii is an obligate intracellular protozoan parasite that causes toxoplasmosis and threatens warm blooded animal and human health worldwide [1]. It is estimated that more than 30% of the global population is reportedly seropositive with *T. gondii*, although the infection rates vary significantly by geographical region [2]. A recent analysis reported antibody positive rates of 8.20 and 8.60% for *T. gondii* in the general population and pregnant women across China, respectively [3]. People with normal immune function may not have typical symptoms and show recessive infection after infection with *T. gondii*, but pregnant women infected with *T. gondii* can lead to premature delivery, abortion, teratosis, stillbirth, etc., and newborn is regularly accompanied by serious eye diseases [1, 4]. For humans with weakened immune function or immunodeficiency, such as AIDS patients, organ transplant recipients, tumor patients, etc., infection with *T. gondii* can cause extreme encephalitis and even death [5, 6]. Therefore, effective, rapid and accurate diagnosis is needed to improve development of treatment approaches, enhance prognosis and control dissemination.

At present, the testing targets for Toxoplasmosis consist of the pathogenic methods, immunological techniques and molecular biological methods [7, 8]. Above all, direct pathogenic diagnosis of *T. gondii* involves the detection of tachyzoites or tissue cysts by direct microscopy or isolation in cell culture. However, pathologic tissue examination or strain isolation are mostly used for diagnosis of animal infection, less frequently for that of human toxoplasmosis [1]. Currently, nucleic acid and specific

antibody assays of *T. gondii* are the most commonly used techniques in clinical settings [9]. Particularly, the serum IgM/IgG antibody test has been widely used as a primary screening method for toxoplasmosis infection. However, the sensitivity and specificity of this method are not very high, and it is easy to produce false positive. In recent years, PCR, nested PCR, and real-time PCR (qPCR) assays have become essential tools for the molecular diagnosis of *T. gondii*. These PCR-based amplification techniques have revealed good sensitivity and specificity, and qPCR with probe hybridization reported to be the most sensitive assay [10]. Nevertheless, widespread clinical application of these techniques has been limited by several factors, including the need for sophisticated instruments, complicated operation and well trained personnel.

To date, several new molecular biological methods that require a uniform incubation temperature have been described, which significantly reduce the reaction time and complexity of nucleic acid amplifications. These isothermal nucleic acid amplification methods, such as the loop-mediated isothermal amplification (LAMP), require only a heating block can maintain a constant temperature rather than an expensive thermocycler [11]. However, the LAMP method is prone to aerosol pollution and the result becomes a false positive. Recombinase aided amplification (RAA) is a new technology for nucleic acid amplification, and it works using four enzymes (UvsX, UvsY, SSB, and polymerase) at a constant temperature [12]. The RAA requires only a simple thermostatic device (constant temperature 37–42°C) and a short reaction time (30 min) [13]. DNA amplification can be detected by agarose electrophoresis (AGE), real-time fluorescence methods and lateral flow dipstick (LFD) [14], which have subsequently improved the sensitivity and specificity of the RAA assay. Among these optimizations, the LFD method is easy to use and its results easily interpretable. It can be used to detect proteins and nucleic acids [15]. Up to now, RAA-LFD has been applied for detection of microbes, such as Newcastle disease virus [16], dengue virus [13], avian infectious laryngotracheitis virus [17], Novel Coronavirus [18], even to identify the genetic sex of *Cynoglossus semilaevis* [14]. CRISPR-Cas systems are widely used for genome editing, in recent years, the trans-cleavage activity of Cas proteins has been discovered [19, 20]. Detection methods based on CRISPR-Cas, which is now also considered a next-generation pathogen detection method, have become established [21, 22]. DNA endonuclease targeted CRISPR trans-reporter and one-hour low-cost multipurpose highly efficient system can detect DNA sequences with high sensitivity and well specificity using CRISPR-Cas13a [23]. Methods such as SHERLOCK (specific high-sensitivity enzymatic reporter unlocking), which typically use target amplification followed by CRISPR-mediated nucleic acid detection have been used to detect SARS-CoV-2 [24].

During toxoplasmosis diagnosis, a series of nucleic acid amplification assays, targeting B1 gene or 529bp repeat sequences, internal transcriptional spacer sequences (ITS-1), as well as 18S rDNA sequences, have been established. While the 529bp repeat sequences showed better sensitivity and specificity for the diagnosis of toxoplasmosis [25]. According to literature available, there is no RAA amplification and LFD detection coupled with CRISPR-Cas13a fluorescence assay was been used for the diagnose of toxoplasmosis. In this study, we targeted the 529bp repeated element of *T. gondii* and designed the crRNA probe with the Cas13a-based detection system in combination with RAA and LFD

methodology for easy visualization. Furthermore, we validated the established RAA-Cas13a-LFD assay by detecting *T. gondii* in genomic DNA extracted from clinical blood samples.

Materials And Methods

Strains and Clinical samples

T. gondii tachyzoites (RH strain), preserved at our laboratory, were aseptically cultured in vitro, by serial passages in Vero cells, as previously described [26]. A total of 267 blood samples from patients clinically at the First Affiliated Hospital of Wannan Medical College were collected between December 2018 to November 2020. The blood samples were divided in two aliquots and placed in the anticoagulant tubes (containing 10µL 0.5µmol /L EDTA) and non-anticoagulant tubes, respectively. The blood in the non-anticoagulant tubes was centrifuged and the serum was aseptically separated for serology with electrochemiluminescence (ECL) assay (Roche, Switzerland). Blood from anticoagulant tubes is used to extract genomic DNA.

DNA extraction

A DNeasy Blood & Tissue Kit (Tiangen Biotech Beijing Co., Ltd., China) was used, to extract genomic DNA from blood samples under the the manufacturer's instructions. All these DNA samples were stored at -20°C until subsequent analysis. Due to the suspected low amount of circulating DNA in these blood samples whose status infection was unknown, each sample was divided into three parts and repeated eluted in order to improve DNA yield and detection rate. Each repliate was then tested and a positive result for any of the sections indicated that the sample was positive.

Construction of standard recombinant plasmids

Previous studies have reported the 529bp repeated element (GenBank AF146527) as the potential optimal targets for *T. gondii* [27]. The 529bp repeated element is a recently discovered target gene, with up to 300 copies, which offers more sensitivity and specificity during detection [27]. PCR products of *T. gondii* 529bp fragment were amplified in the positive tachyzoites of *T. gondii*, and electrophoresed, excised, purified, and cloned into the pMD™19-T vector (Takara). The positive clones were sequenced directionally (ABI 3730). The positive recombinant plasmids of *T. gondii* are kept in our laboratory (Wannan Medical College, China). It is the recombinant plasmids used as standard nucleic acids were quantified with a NanoDrop ND1000 spectrophotometer. Then the recombinant plasmids were prepared with a dilution range 1 ng/µL-1×10⁻⁹ ng/µL as a standard control and stored at -20°C until needed.

Design of primer and crRNA probe

Since success of RAA amplification depends on designing the ideal primers for target the gene, we employed the online primer designing software and designed 5 pairs of specific oligonucleotide primers targeting the 529bp repeated element of *T. gondii*. The primers annealing temperature are 54–67°C with the length about 30-35bp, and the amplicon size is (100-300bp) (Table 1). Specific crRNA probes were

designed target the specific primer sequence fragment (Table 1). The DNA probes were prepared into RNA by in vitro transcription, according to the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, America). There should be paid to the 5' end accessory T7 RNA polymerase promoter sequence during probe design. The transcript (crRNA probe) was purified using RNAXP magnetic beads and the concentration of the product was determined by Qubit nucleic acid concentration detector (Thermo, America). All primers were synthesized by TsingKe Biotech (Beijing TsingKe Biotechnology, Beijing, China).

Table 1
Primer sequence for RAA-Cas13a-LFD assay.

Primer	Sequence(5'to3')	Amplicon sizes (bp)	Description
TOX-F1	ACTACAGACGCGATGCCGCTCCTCC	283	Candidates for RAA primers that were screened in this study. TOX-F1/TOX-R1 pair was used for follow-up experiment experiments
TOX-R1	TGTCTCCCTCGCCCTCTTCTCCACT		
TOX-F2	ACACCGGAATGCGATCCAGACGAGA	126	
TOX-R2	GTCCAAGCCTCCGACTCTGTCTCCC		
TOX-F3	ATATCAGGACTGTAGATGAAGGCGAGGGT	176	
TOX-R3	CGTCTCGTCTGGATCGCATTCCGGTGTCT		
TOX-F4	TAGATGAAGGCGAGGGTGAGGATGAGGGG	121	
TOX-R4	CTCCAGGAAAAGCAGCCAAGCCGGAAACA		
TOX-F5	AAGATGTTTCCGGCTTGGCTGCTTTTCCTG	162	
TOX-R5	CCGACTCTGTCTCCCTCGCCCTCTTCTCCA		
T7 promoter	GAAATTAATACGACTCACTATAGGG		Promote the transcription of amplified DNA to RNA in vitro transcription.
Probe1	CGUCUCGUCUGGAUCGCAUUCGGUGUC		Expected sequences of guide RNA obtained from in vitro transcription. Probe1 was used for follow-up experiment experiments.
Probe-2-5-a	UUCUCUCCGCCAUCACCACGAGGAAAGC		
Probe-2-5-b	CAAUUCUCUCCGCCAUCACCACGAGGAA		
Probe-3-4-a	CUCCGACUCUCGUCGCUUCCCAACCACG		
Probe-3-4-b	CCGACUCUCGUCGCUUCCCAACCACGCC		
Lateral flow reporter	FAM/mArArUrGrGrCmAmArArUrGrGrCmA/Bio		Trans cleavage reporter for Cas13a

Establishment of RAA assay

The basic RAA reactions were achieved by the Basic RAA kits (Anhui Microanaly Genetech, Hefei, China). The reaction buffer was premixed according to the following formula: 25µL of rehydration buffer, upstream primers (10µmol/L) 2µL, downstream primers (10µmol/L) 2µL, 25×SYBR Green 1µL,

recombinant plasmids (1ng/ μ L) 2 μ L, MgOAc solution (280nmol/L) 2.5 μ L, and 17.5 μ L of nuclease-free water. The aforementioned reaction tube was put into fluorescent quantitative PCR instrument to amplify for 40min at 37°C. The amplified products were analyzed Qsep100 biological analyzer (BIOptic, Taiwan) by collecting the fluorescence.

Establishment of RAA-Cas13a-LFD assay

Here, targeted detection was used gene editing technology based on nucleic acid amplification and Cas13a-mediated collateral cleavage of a reporter RNA, allowing for real-time detection of the target. The 529bp target gene of *T. gondii* was amplified by RAA, T7 RNA polymerase transcription of amplified DNA to RNA and detection of target RNA by Cas13a collateral RNA cleavage mediated release of reporter signal. The fluorescence reporter signal can be detected by LFD, LFD is an endpoint assay, with lateral flow strips exposed to the reaction mixture post incubation (Fig. 1).

The RAA-Cas13a-LFD reaction formula was as the following: LwCas13a nuclease (45nmol/L) (Anhui Microanaly Genetech, Hefei, China)), Lateral flow reporter molecule (125nmol/L) (Integrated DNA Technologies, Iowa, USA), RNase inhibitor (1 μ L) (New England Biolabs, America), ATP (1mM), GTP (1mM), UTP (1mM), CTP (1mM) and T7 polymerase (0.6 μ L) were mixed to the total system 8 μ L. Then, 1 μ L crRNA probe (30ng/ μ L) and 1 μ L of RAA products were added into 8 μ L of aforementioned mixture and mixed thoroughly. It is recommended to incubation at 37°C for 40 minutes. Add 20 μ L diluent buffer to the 10 μ L of the reaction and mix well. The lateral-flow dipsticks were insert into the mixture for 3–5 minutes. and results were recorded until the positive control line was visualized.

Results explanation of RAA-Cas13a-LFD assay

Cas13a detection was adapted for lateral flow detection (LFD), with an important alteration: the Lateral flow reporter was replaced with FAM-ssRNA-Biotin reporter (FB reporter) [28, 29]. Without trans cleavage, the FB reporter will remain intact and its biotinylated end can be captured by streptavidin at the control line. Anti-FAM antibody conjugated to gold nanoparticles can then bind the exposed FAM moiety, resulting in the color deposit on the control line. It was negative if only control line (red band) appeared, indicating that there is not the amplification fragment of the target gene in the sample (Fig. 2). On the other hand, if the FB reporter has undergone trans cleavage by Cas13a, a portion of reporter molecules will contain biotin, but not FAM. As a result, fewer FAM ends will be present at the control line, and a higher amount of antibody-conjugated gold nanoparticles will be able to travel further to deposit on the test line. It was positive when test line and control line both appeared or only test line red band appears, indicating that the target gene of *T. gondii* to be detected in the sample.

Evaluation of RAA-Cas13a-LFD specificity and sensitivity

In order to determine the analytical specificity of the RAA-Cas13a-LFD assay developed in this study, the nucleic acids of human blood, *Ascaris lumbricoides*, *Digamma interrupta*, *Entamoeba coli*, *Fasciola gigantica*, *Plasmodium vivax*, *Schistosoma japonicum*, *Taenia solium* and *Trichinella spiralis* were used

as templates for the RAA reactions. The sensitivity of the RAA-Cas13a-LFD assay was assessed using 10-fold serial dilutions of the recombinant plasmids ranging from 1 ng/μL-1×10⁻⁹ ng/μL. All experiments were carried out in duplicate, and the recombinant plasmid was the positive control and ddH₂O was the negative control. Different RAA products were directly analyzed by lateral-flow dipsticks.

To verify the analytical specificity and sensitivity of the RAA-Cas13a-LFD assay developed in this study, a real-time PCR (qPCR) also performed using the forward primer, reverse primer, and probe primer target the the same 529bp gene. The sequence of forward primer, reverse primer, and probe primer are AGACGAGACGACGCTTTCC, GCATCTGGATTCTCTCTACC, and CGTCCAAGCCTCCGACTCTGTCTC, respectively. qPCR reactions were performed in 20 μL reaction volumes, comprising 10μL 2×Master Mix (Roche, Switzerland), 10μM of each forward and reverse primers, 10μM of probe primer, and 2μL of the template. Amplification was performed as follows: initial denaturation at 95°C for 30s; followed by 40 cycles of denaturation at 95°C for 10 s, primer annealing at 60°C for 30s; dissociation at 95°C for 15s, 60°C for 60s, and 95°C for 15s. The products of qPCR were visualized according to amplification curve generated by the LightCycler 96 qPCR instrument (Roche, Switzerland).

Application of the RAA-Cas13a-LFD assay for clinical samples

We tested the established RAA-Cas13a-LFD method for detection of *T. gondii* in blood samples collected from patients of Clinical Laboratory. A total of 267 blood samples were simultaneously tested with serology ECL assay, qPCR and the RAA-Cas13a-LFD assay for detection of *T. gondii*. To evaluate the validity of establishment RAA-Cas13a-LFD assay, blood samples were all to amplify the 529 gene for detection of *T. gondii* by qPCR assay.

Results

Optimization of RAA primers

There are five pairs of primers were designed for 529bp gene region in accordance with the requirements of RAA nucleic acid amplification (Table 1). After the products were amplified by RAA nucleic acid amplification at 37 °C for 40 minutes, they were screened by fluorescence detection and capillary electrophoresis.

According to the primers melting curves (Fig. 3), the primer pairs of 1 and 3 have a single high peak, and there is no nonspecific amplification. The size of amplified product fragment of primer pair 1 and 3 are about 283 bp and 176 bp, respectively, they are consistent with the expected target fragment; Furthermore, the total amount of products of primer pair 1 and 3 are relatively high (Fig. 4a and 4c, Table 2). The primer pairs of 2 and 5 have many impurity peaks in the melting curve, resulting in nonspecific amplification and have a low total amount of amplified products (Fig. 3, Fig. 4b and 4e, Table 2). Although the primer dissolution curve of primer 4 is a single peak, and there are no nonspecific amplification. While the total amount of product is low, indicating poor amplification efficiency (Fig. 3, Fig. 4d, Table 2). In brief, based on the results of the melting curve and the peak of the amplified product,

the amplification efficiency of primer pairs of 1 and 3 is relative better. Hence, the primer pairs of 1 and 3 were used in the follow up probe optimizing test.

Table 2
Amplification products of 5 pairs of primers by Qsep100 bioanalyzer test

Primer pair	Size of main peak (bp)	Total amount of target product(ng)	Total amount of nonspecific amplification products(ng)	Target product ratio(%)
Primer pair 1	283	990	0	100
Primer pair 2	126	72	90	44
Primer pair 3	176	560	0	100
Primer pair 4	121	150	0	100
Primer pair 5	162	160	164	49

Optimization of crRNA probe primers

After the positive plasmid is amplified by RAA isothermally, fluorescence method is used to optimize the combination of primer and probe. The amplification efficiency of the primer-probe combination was evaluated by the fluorescence amplification curve. According to the fluorescence amplification curve (Fig. 5a), the fluorescence value of positive plasmid group based on the Primer1 + Probe1 combination has a significant difference in compared with the negative control (NTC) group, indicating that the detection result is positive and the amplification efficiency is well; while there are significant amplification based on the Primer1 + Probe3-4-a and Primer3 + Probe3-4-a combination and the consistency is poor (Fig. 5b); similar results, the amplification curve of the Primer1 + Probe3-4-b has poor consistency, and the fluorescence value of Primer3 + Probe3-4-b has no significant difference compared with the NTC group, the amplification efficiency is extremely lower (Fig. 5c). In summary, the Primer1 + Probe1 combination has the best amplification efficiency, which was used in the subsequent RAA-Cas13a-LFD experiments.

Optimization of the RAA-Cas13a-LFD assay conditions

The preferred time for the RAA-Cas13a-LFD reaction were evaluated. First, RAA basic reactions were carried out at 37 °C for 40 minutes in a heating block. Results of the amplification showed that the target DNA could be successfully amplified. A body temperature of 37 °C was chosen as the optimal RAA-Cas13a-LFD temperature. Take the plasmids with concentrations of 1×10^{-5} ng/μL, 1×10^{-6} ng/μL and 1×10^{-7} ng/μL as templates; the amplification times are 20min, 30min and 40min for the reaction. The results showed that when the RAA-Cas13a-LFD reaction was amplified for 20 minutes, the detection limit

of plasmid concentrations was 1×10^{-5} ng/ μ L, and when the RAA-Cas13a-LFD reaction was amplified for 30 minutes and 40 minutes, the detection limit of plasmid concentrations was 1×10^{-6} ng/ μ L (Fig. 6). The detection limit of amplification yielded did not change with the increase of time from 30min to 40min, so 30 minutes was chosen as the RAA-Cas13a-LFD reaction time in this study.

Sensitivity of the established RAA-Cas13a-LFD assay

The sensitivity of the RAA-Cas13a-LFD assay was determined by using serial 10-fold dilutions of a positive control template (positive recombinant plasmid of *T. gondii*). These dilutions ranged were from 1 ng/ μ L to 1×10^{-9} ng/ μ L, with ddH₂O included as a negative control. The amplified reaction was performed for 30min, and the lowest detection concentration of RAA-Cas13a-LFD method was determined. As shown in Fig. 7a, there were the corresponding visible test lines when the concentration of recombinant plasmids was $> 1 \times 10^{-6}$ ng/ μ L. We also performed a qPCR as described before. It can be seen that the minimum detection limit of the qPCR is 1×10^{-8} ng/ μ L (Fig. 7B). The results indicated that the limit of RAA-Cas13a-LFD assay for *T. gondii* is slightly less sensitive compared with qPCR in the level of detection limit.

Specificity of the established RAA-Cas13a-LFD assay

We tested specificity of RAA-Cas13a-LFD and qPCR methods using genomic DNA samples from human blood, *Ascaris lumbricoides*, *Digramma interrupta*, *Entamoeba coli*, *Fasciola gigantica*, *Plasmodium vivax*, *Schistosoma japonicum*, *Taenia solium* and *Trichinella spiralis*. The *T. gondii* positive plasmid was used as a positive control, and ddH₂O was used as a negative control. Only *T. gondii* positive plasmids showed amplification using both methods, and produced visible test bands (RAA-Cas13a-LFD) and amplification curves (qPCR). While the other DNA templates showed no signals (Fig. 8), demonstrating that the established RAA-Cas13a-LFD method has good specificity.

Clinical performance of the established RAA-Cas13a-LFD assay

To evaluate the clinical performance of the *T. gondii* RAA-Cas13a-LFD assay, a total of 267 clinical blood samples (137 males and 130 females) were collected from patients of Laboratory Medicine and tested using ECL, qPCR and the established RAA-Cas13a-LFD methods, respectively. The results showed that the positive rates of *T. gondii* in the blood samples are various in different methods (Table 3). Higher positive rates were detected by RAA-Cas13a-LFD (1.50%, 4/267) than by ECL-IgM (1.12%, 3/267). Compared with qPCR, there was a consistent positive rate between qPCR and RAA-Cas13a-LFD assays (Fig. 9, Table 3). Indicating that the established RAA-Cas13a-LFD assay offers excellent performance for detecting *T. gondii* as the same as qPCR in clinical setting.

Table 3
Comparison of ECL, qPCR and RAA-Cas13a-LFD assay for detection of *T. gondii* in blood samples

Test methods	Blood samples (n = 267)	
	positive	negative
ECL IgG	34 (12.73%)	233 (87.27%)
IgM	3 (1.12%)	264 (98.88%)
qPCR	4 (1.50%)	263 (98.50%)
RAA-Cas13a-LFD	4 (1.50%)	263 (98.50%)

Discussion

Thus far, several nucleic acid-based detection platforms have been developed for *T. gondii*, including traditional PCR, nested PCR, qPCR and LAMP coupled with LFD. Although these methods offer good sensitivity, they are limited by various constraints such as susceptibility to false positives and lack of compatibility with point-of-need applications [7]. LAMP is characterized by a streamlined protocol and exemplary detection limit of 1 copy of *T. gondii* DNA per reaction, but spurious amplification can occasionally arise and is prone to aerosol pollution [5]. RAA-LFD is to perform isothermal amplification of the target gene or specimen by the RAA method and then combine with LFD technology, LFD is a technology that finally realizes the visual analysis of amplified products. This technology does not require expensive equipment, can quickly and effectively amplify the target fragment under constant temperature conditions, and the result of the reaction can be judged by the naked eye. Whereas RAA alone was not sensitive enough to detect low levels of target [23]. In multiple Cas family members, including Cas13, Cas12 and Cas14 effector, cutting the target nucleic acid can trigger the cleavage of irrelevant single-strand DNA (ssDNA) or single-strand RNA (ssRNA). This collateral cleavage has been exploited for nucleic acid detection [30–32]. CRISPR-Cas13a (formerly C2c2), a Type VI Class 2 CRISPR-Cas effector, is a single-component enzyme targeting single-stranded RNA with a guide RNA. Binding with a complementary ssRNA will activate its targeting and general ssRNase activity [19, 23], the latter responsible for collateral ssRNA cleavage. In the specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) platform, quenched fluorophore is added to the collateral substrate, Cas13a system cleaves a nucleic acid reporter and generates a detectable signal, thus enabling target RNA detection [28]. The fluorescence signal is monitored using the spectroscopy reader, such as LFD. The CRISPR-Cas13a system has been employed for nucleic acid detection of a variety of pathogens, including SARS-CoV-2, Zika virus, dengue virus, among others [23].

Therefore, for nucleic acid detection of *T. gondii* in this study, a RAA is used to amplify target DNA, which is then transcribed by T7 RNA polymerase promoter into RNA for subsequent LwCas13a detection, then the fluorescence signal is monitored by LFD observation with naked eyes. A new type of RAA-LFD combined Cas13a detection method for *T. gondii* based on 529 bp gene was successfully established. It

had no cross-reactivity with other parasites and could consistently detect 1×10^{-6} ng/ μ L of DNA per reaction. The established RAA-Cas13a-LFD assay can complete the amplification reaction at a constant temperature of 37°C for less than 2 hours for naked eye visualization.

With the detection limit of 1×10^{-6} ng/ μ L per reaction, the sensitivity of RAA-Cas13a-LFD assay is slightly less sensitive than qPCR, which could explain the disagreement between two approaches at the lowest target concentration of *T. gondii* [33]. In addition, qPCR and RAA-Cas13a-LFD are suitable for different environmental and laboratory conditions to detection of *T. gondii*. These advantages combined render RAA-Cas13a-LFD a strong option for routine surveillance in resource-limited settings. Nonetheless, RAA-LFD combined Cas13a assay developed in this work should still be sensitive enough to permit timely detection of *T. gondii* among the clinical samples; the positive rate of RAA-Cas13a-LFD was consistent with that of qPCR among the clinical samples. It was found that qPCR and RAA-Cas13a-LFD methods were all detected 4 positive cases of 267 clinical samples, while ELISA method (IgM⁺) only detected 3 cases of positive specimens. The assay established in this study indicated that the sensitivity of RAA-Cas13a-LFD method is higher than conventional ECL detection.

Through the *T. gondii* can be detected from the clinical blood samples within 2h by naked eye observation under a constant temperature heating block in this study, but it is on the premise of the genome DNA from the blood sample has been extracted. The effective acquisition of nucleic acid detection is the basis of simple and fast RAA-Cas13a-LFD assay in the current study. Therefore, further development is needed especially for simply DNA acquisition techniques from blood sample. A rapid and sensitive RAA assay to detect *Bordetella pertussis* using the DNA obtained by boiling clinical samples of respiratory secretions has been reported [34]. Additionally, compared with traditional PCR technology, CRISPR/ Cas13-based nucleic acid detection technology has higher sensitivity, better specificity, and does not require expensive equipment and professional operators [35]. While compared with qPCR in this study, the detection limit of blood-extracted *T. gondii* DNA RAA-Cas13a-LFD assay should be fully determined through screening of primer and probe combination, to achieve a more similar sensitivity to qPCR.

Conclusions

In conclusion, the developed RAA-LFD combined Cas13a assay has high specificity and sensitivity, and is visual, rapid and reliable for *T. gondii* detection. A heating instrument cannot be procured; it may even be possible to exploit body heat for incubation in this establishment assay. Furthermore, the assay could be adapted into an LFD format that does not demand any visualization instrument to achieve visual detection of *T. gondii*. It has the advantages of simple operation, fast response, high sensitivity, good specificity, and visualization of reaction results. It is very suitable for on-site testing and has a good prospect in clinical applications.

Declarations

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Authors' contributions

JZ and FF conceived and designed the experiments; JZ, FF, QX, ZZ and MZ performed the work and acquired the data. JZ and YL wrote the manuscript. QX provided methodological input, and contributed to writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article

Ethics approval and consent to participate

All aspects of the study were performed in accordance with national ethics regulations and approved by the Medical Ethics Committee of Wannan Medical College.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Schematic of *T. gondii* detection with RAA-Cas13a-LFD assay. Steps include: amplification of target DNA with RAA; T7 RNA polymerase transcription of amplified DNA to RNA; target recognition the target RNA with Cas13a-crRNA, collateral cleavage of fluorophore-quencher reporter (i.e. lateral flow reporter) and release fluorophore signal; visualization of the unleashed fluorescence signal by lateral flow dipstick.

Melt Curve Plot

Figure 2

Results explanation of RAA-Cas13a-LFD assay.

Positive result	Negative result	Invalid result
<div> <div>T</div> <div>C</div> <div> <div></div> <div></div> </div> </div>	<div> <div>C</div> <div></div> <div> <div></div> <div></div> </div> </div>	<div> <div>T</div> <div>C</div> <div> <div></div> <div></div> </div> </div>
C and T appear or T appears alone	C appears alone	Neither C nor T appear

Note: T means test line; C means control line.

Figure 3

The melting curves of 5 pairs of primers.

Figure 5

Fluorescence amplification curve of primer and probe combination. a The fluorescence amplification curve of Primer1+Probe1 combination, the fluorescence value of amplification is very high in the positive plasmid control of *T. gondii* for three repeats. b The fluorescence amplification curve of Primer1+ Probe3-4-a and Primer3+ Probe3-4-a, there are no significant amplification, and the consistency is poor for three repeats. c The fluorescence amplification curve of Primer1+ Probe3-4-b and Primer3+ Probe3-4-b, there

are no significant amplification, and the amplification consistency of Primer1+ Probe3-4-b is poor for three repeats. Abbreviations: PP, positive plasmid control; NTC, negative control.

Figure 6

Optimization result of RAA-Cas13a-LFD amplification time. Strips: 1. 1×10⁻⁵ ng/μL; 2. 1×10⁻⁶ ng/μL; 3. 1×10⁻⁷ ng/μL; NTC, negative control (ddH₂O).

Figure 7

The sensitivity of RAA-Cas13a-LFD and qPCR detection methods for *T. gondii*. a Visual inspection of RAA-Cas13a-LFD. b Curves for qPCR. Strip and curves: 1-10: plasmid copy number diluted to 1 ng/ μ L - 1×10^{-9} ng/ μ L, respectively; NTC, negative control (ddH₂O).

Figure 8

The specificity of RAA-LFD and qPCR detection methods for *T. gondii*. **a** Visual inspection of RAA-Cas13a-LFD. **b** Curves for qPCR. Strip and curves: 1. Human blood; 2. *Ascaris lumbricoides*; 3. *Diagramma interrupta*; 4. *Entamoeba coli*; 5. *Fasciola gigantica*; 6. *Plasmodium vivax*; 7. *Schistosoma japonicum*; 8. *Taenia solium*; 9. *Trichinella spiralis*; Abbreviations: PP, Positive plasmid of *T. gondii*; NTC, negative control (ddH₂O)

a PP 1 2 3 4 5 6 7 8 NTC

b

Figure 9

Detection of blood samples of RAA-Cas13a-LFD and qPCR methods for *T. gondii*. a Visual inspection of RAA-Cas13a-LFD. b Curves for qPCR. Strip and curves: 1-4: positive sample; 5-8: negative samples; Abbreviations: PP, Positive plasmid of *T. gondii*; NTC, negative control (ddH₂O)

Supplementary Files

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