

# Rapid Detection of *Toxoplasma gondii*. Prevalence Investigation of *T. gondii* Infection Among Stray Cats and Dogs in Zhejiang Province Based on a New Loop-Mediated Isothermal Amplification-Lateral-Flow-Dipstick (LAMP-LFD) Device

**Yangji Xue**

Zhejiang Academy of Medical Sciences

**Shaohong Lu**

Zhejiang Academy of Medical Sciences

**Qingming Kong**

Zhejiang Academy of Medical Sciences

**Haojie Ding**

Zhejiang Academy of Medical Sciences

**Jianzu Ding**

Zhejiang Academy of Medical Sciences

**Bin Zheng**

Zhejiang Academy of Medical Sciences

**Qunbo Tong**

Zhejiang Academy of Medical Sciences

**Xunhui Zhuo**

Zhejiang Academy of Medical Sciences

**Chengzuo Xie**

Zhejiang Academy of Medical Sciences

**Di Lou**

Zhejiang Academy of Medical Sciences: Zhejiang University School of Medicine

**Hangjun Lv** (✉ [lhj@zjams.com.cn](mailto:lhj@zjams.com.cn))

Zhejiang Academy of Medical Sciences: Zhejiang University School of Medicine

---

**Research**

**Keywords:** *T. gondii*, Diagnosis, 529-RE, LAMP, LFD, prevalence

**Posted Date:** September 8th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-69332/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

**Background:** *Toxoplasma gondii* (*T. gondii*) is worldwide spread caused Toxoplasmosis threatening warm-blooded animal and human health, especially for immunodeficient population and pregnant women. Simple and applicable diagnostic methods are urgently needed for the prevention of toxoplasmosis. The molecular diagnosis of *T. gondii* infection generally requires high technical skills, sophisticated equipment and a controlled lab environment.

**Methods:** In this study, we developed a loop-mediated isothermal amplification-lateral-flow-dipstick (LAMP-LFD) assay that specifically targets the 529 bp for the detection of *T. gondii* infection in a new kind of portable device, which is universal, fast, user-friendliness, experimental sensitivity and low risk of aerosol contamination.

**Results:** The detection limit of the LAMP-LFD assay is 1 fg of *T. gondii* DNA and no cross-reaction with other parasitic pathogens including *Leishmania donovani*, *Plasmodium vivax*, *Cryptosporidium parvum*, etc. In total, 318 stray cat and dog blood samples were collected from Deqing, Wenzhou, Yiwu, Lishui and Zhoushan cities in Zhejiang province, Eastern China. The current infection prevalence of *T. gondii* was 4.76% and 4.69% in stray cats and dogs respectively, detected by LAMP-LFD device.

**Conclusions:** In conclusion, the established LAMP-LFD was an efficient and avoidable aerosol-contaminated device that can detect 1 fg genomic DNA of *T. gondii*, and suitable for *T. gondii* detection in the basic medical institution and even in field areas.

## Introduction

*Toxoplasma gondii* (*T. gondii*) is an obligate intracellular protozoan parasite that is globally distributed and can infect a wide variety of warm-blooded animals including humans and causing Toxoplasmosis [1, 2]. *T. gondii* infections are distributed worldwide, with an estimated one-third of the global population being seropositive for *T. gondii* but significant geographical variations in infection rates[3]. A recent analysis showed that the antibody positivity rate for *T. gondii* in the general population of China was 8.20% and 8.60% for pregnant women[4, 5]. *T. gondii* can invade all nucleated host cells and obtain the vital nutrients needed to replicate itself[6]. It is now widely believed that the foodborne transmission of *T. gondii* for human occurs by the ingestion of undercooked meat containing tissue cysts, consumption of water or food contaminated with toxoplasmosis oocyst, etc[7, 8], meanwhile, some scholars propose that arthropods such as ticks play a role in the spread of *T. gondii* [9, 10]. In general, primary infection of *T. gondii* in immunocompetent individuals is usually subclinical such as some symptoms of malaise, fever, myalgias, and isolated cervical or occipital lymphadenopathy, and then the cysts are formed in the brain, skeletal muscle, heart, or other organs[11]. As an important opportunistic pathogen in immunocompromised patients, a global meta-analysis suggests that *T. gondii* infection rate is higher in patients with AIDS, malignancies, organ transplant, upon this severe immunosuppression, the *T. gondii* tachyzoites derived from activated encysted bradyzoites replicate rapidly and result in more severe

clinical presentation of encephalitis, pneumonia, retinochoroiditis, other disseminated systemic diseases and even death[12, 13]. Furthermore, pregnant women infected with *T. gondii* before or just during pregnancy can result in miscarriage, stillbirth, fetal abnormalities, neural and neurocognitive deficits, even congenital transmission to the fetus if the *T. gondii* cross the placenta[14]. Now, the treatment of toxoplasmosis in the active stage relies on the combination drugs of pyrimethamine and sulfadiazine(pyr-sulf), but the spiramycin for acute maternal infections is more suitable and pyr-sulf for established fetal infection[15]. Effective, rapid and accurate diagnosis is therefore essential and desirable for initiating appropriate treatment and achieving a good prognosis[16].

The pathogenetic examination of *T. gondii* infection mainly includes direct microscopy, trophozoite isolation culture and cyst examination, but it is difficult to confirm the diagnosis, and it is mostly used in animal infection diagnosis or strain isolation, as well as human pathological tissue examination under special circumstances. Currently, nucleic acid, serum circulating antigen, and specific antibody assays of *T. gondii* are more commonly used in clinical settings[17]. In particular, the serum IgM/IgG antibody test is widely used as a primary screening method for toxoplasmosis infection. However, this method may fail to detect specific anti-*T. gondii* antibody during the active phase of infection, because these antibodies can only be produced after several weeks of parasitemia[18]. In recent years, PCR, real-time PCR and nested-PCR strategies based on polymerase chain reaction have become essential tools for the molecular diagnosis of *T. gondii*. These PCR amplification techniques showed good sensitivity and specificity in the molecular detection of *T. gondii*, and the real-time PCR with probe hybridization has been reported to be the most sensitive assay[19]. Nevertheless, factors such as the need for sophisticated instruments and well-trained personnel limit the widespread clinical application of these techniques.

The loop-mediated isothermal amplification(LAMP) technique has attracted much attention since its discovery because of its simpler amplification conditions and high-efficiency amplification. The technique is based on strand displacement activity *Bst* DNA polymerase with and two pairs of specially designed primers, at a constant temperature of around 60–65 °C, and a set of amplification products consisting of stem-loop structures containing repetitive target sequences forms[20]. The LAMP product can be judged by the turbidity of the resulting magnesium pyrophosphate by visual inspection, or by the addition of a fluorescent dye to the reaction system to make the product visible under UV light[21]. The advantages of LAMP technology over traditional PCR put it at the forefront of research in the search for new diagnostic tools for parasitic diseases[18]. In toxoplasmosis diagnosis, a series of LAMP-based assays targeting B1 gene or 529 bp repeat sequences, internal transcriptional spacer sequences (ITS1), and 18S rDNA sequences have been established. Besides, the detection of LAMP products was further optimized by combining probe hybridization[22], ELISA[23], and lateral flow dipstick (LFD)[24], that improves the sensitivity and specificity of LAMP assay. Among these, LAMP-LFD is one of the optimal detection methods and has been validated in the detection of parasites and microbes, such as *Mycoplasma ovipneumoniae*[25], *Toxoplasma gondii*[26], *Babesia bovis* and *Babesia bigemina*[27], canine parvovirus[28], and African trypanosome[29]. LAMP-LFD is based on the principle that biotin-primers biotinylate the amplification product and fluorescein isothiocyanate (FITC)-labeled probes de-hybridize so that the product is simultaneously double-labeled and then captured by anti-FITC antibodies

on the lateral flow dipstick. Conventional LAMP-LFD method requires the reaction tubes to be opened to add the reaction product to the LFD after the LAMP amplification completed. Yet, the efficient amplification mechanism in LAMP experiment makes it highly sensitive to aerosol contamination from previous LAMP reactions and serves as a template for repeated amplification, leading to inaccurate false-positives results[30, 31]. In order to address this problem, we have designed a simplified, portable, closed LAMP-LFD format. In the present study, we targeted the 529-repeated element of *T. gondii*, and developed a new sensitive and simple assay based on LAMP-LFD in a hermetic device that for *T. gondii* detection. This device performs well in a simply regular laboratory water bath and can simply read-out. The performance of the LAMP-LFD device is identified by the *T. gondii* tachyzoite genomic DNA and stray cats and dogs blood samples collected from Zhejiang province, China.

## Materials And Methods

### Ethics

This study was carried out in strict conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals according to the Animal Ethics Procedures and Guidelines of the Chinese National Institutes of Health. The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Academy of Medical Sciences (Approval ID: 2018 - 102).

### Strains and samples

The *Toxoplasma gondii* tachyzoites (RH strain) preserved by our laboratory were used in this study. *T. gondii* tachyzoites were cultured in vitro under standard procedures by serial passages in Vero cell and prepared as described previously[32]. The stray dogs and cats blood sample were provided by animal protection base of Zhejiang Small Animal Protection Association. A total of 318 blood samples were collected from Zhoushan, Deqing, Lishui, Yiwu, Wenzhou respectively, Zhejiang province. For nucleic acid extraction, each blood sample was anticoagulated. All the blood samples were collected by experienced staff from animal hospital and stored at 4°C until been used.

### DNA extraction and serum separation

As positive control, genomic DNA was extracted from *Toxoplasma gondii* RH strains according to the introduction of Animal Genomic DNA Quick Extraction Kit (Beyotime, Shanghai, China). The anticoagulant blood sample was extracted DNA by magnetic beads adsorption using KBM Blood Genomic DNA Extraction Kit (KBM, Hangzhou, China). The concentration of genomic DNA extracted was evaluated on NanoDrop spectrometry (Thermo Fisher Scientific, MA,USA), then stored at -20°C until use..

### Design of primers and probe

According to reported of molecular tests on *T. gondii*, it's proved that B1 gene (GenBank AF179871) and 529-bp repeated element (GenBank AF146527) are the potential optimal targets. B1 gene, which is used in the molecular detection extensively of *T.gondii* previously, has 35 copies in the *T.gondii* genome[33].

529-bp repeated element, as the newly discovered target gene, has up to 300 copies therefore been more sensitively and specifically for detection[34]. Therefore, we target the 529-bp repeated element for *T. gondii* detection in our study. The success of LAMP amplification depends on ideal primers target the gene. For the LAMP assay targeting the 529-bp repeated element of *T.gondii*, we design a set of specific oligonucleotide primers on an online LAMP primer designing software Primer Explorer V3 (<http://primerexplorer.jp/e/>;). The forward inner primer FIP was labeled with biotin in the 5' end and the probe labeled with fluorescein isothiocyanate (FITC) was designed between primers B1c and B2 for molecular hybridization detecting FITC-biotinylated LAMP product (Figure 1). The primer sequences used in our experiment are listed in Table 1.

Table.1.  
The specific primers of PCR and LAMP used in this study.

Primers	Sequences(5'→3')	Sizes of amplicons(bp)
LAMP		202
F3	ACGAGAGTCGGAGAGGGA	
B3	TGGATTCCTCTCCTACCCCT	
FIP(F1c-F2)	GGATCGCATTCCGGTGTCTCTTAAGATGTTTCCGGCTTGGC	
BIP(B1c-B2)	GACGACGCTTTCCTCGTGGTCAAGCCTCCGACTCTGTCT	
FITC-Probe	FITC-GGCGGAGAGAATTGAAGAGTGGAGAA	
PCR		
F	ACGAGAGTCGGAGAGGGA	202
R	TGGATTCCTCTCCTACCCCT	

## LAMP

The LAMP was performed according to the method reported previously[20, 31, 32]. The forward inner primer FIP was labeled with biotin in the 5' end and the probe for detecting biotinylated LAMP product was designed between primers B1c and B2 and labeled with fluorescein isothiocyanate (FITC). A total volume of 25 µL optimized LAMP reaction contains 2-4 µL of the extracted DNA, 12.5 µL of 2× reaction mix buffer(1.6 M betaine, 40mM Tris-HCl (pH 8.8), 20 mM KCl, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 0.2% Tween 20), 5 pmol each of the F3 and B3primers, 40 pmol each of the BIP and biotin-FIP primers, 1 µL of Bst 2.0 WarmStart<sup>®</sup> DNA polymerase (New England Biolabs, Beijing, China), 8.4 mM MgSO<sub>4</sub> (New England Biolabs, Beijing, China), 1.2 uM dNTPs (New England Biolabs, Beijing, China). According to the procedure optimized by our laboratory, the LAMP amplification carried out at 65 °C for 1 h in a constant temperature water bath. For the identification of LAMP product, 1.5% agarose gel electrophoresis was performed. In a

comparative study, 1 uL SYTO13 (Thermofisher, Beijing, China) was used as the fluorescent dye for real-time LAMP, the amplification was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The amplification products were determined by the acquisition of the fluorescent signal.

### **LAMP-Lateral-Flow-Dipstick (LAMP-LFD)**

To detect the products of LAMP, a universal rapid detection equipment LAMP- Lateral-Flow-Dipstick (LAMP-LFD) was designed by combining the LAMP reaction and lateral flow dipstick (Figure 2.A). The integrated equipment has connected micro-amplify reaction tube for LAMP reaction and another tube containing nucleic acid dilution buffer with the lateral-flow-dipstick module for LAMP product capture, and the LFD detection module consists of a plastic grooved pedestal and a hermetic plastic cover that contains a visualization window and two connectors. The lateral-flow-dipstick, which set on the plastic grooved pedestal, is composed of a sample pad, application pad, test line, control line, and water-absorbing pad, that the application pad, test line, and control line covered with gold-streptavidin(SA) conjugates, immobilized anti-FITC mouse monoclonal antibody and biotin respectively. Before starting the assay, the user only needs to connect the micro-amplify reaction tube with the reserved connector after the LAMP reaction mixture adding the sample to be detected (Figure 2.B). Put the LAMP-LFD equipment in a constant temperature water bath at 65 °C for 1 h to accomplish the LAMP reaction, then biotinylated FIP primer and FITC-labeled probe in the LAMP reaction mixture made the biotin and FITC conjunct to the ends of amplifying products stem-loop structure, respectively. For the desired product detection after LAMP reaction accomplished, turn over the equipment, and FITC-biotinylated nucleic acid products mix with the nucleic acid dilution buffer, the mixture flow toward the lateral-flow-dipstick, combine with Gold-streptavidin (SA) conjugates forming a triple-labeled complex when flow through the application band, and then it moves up the strip and is captured by the immobilized anti-FITC antibody (test line). The biotinylated FIP primer binds to the Gold-SA conjugates to form a double complex without FITC and is trapped at the immobilized biotin (control line) (Figure 2.C). Read-out the positive result via both test line and control line are visible through the read-window on the cover. Conversely, only control line visible means negative result.

### **The specificity of LAMP-LFD**

Same LAMP protocol and procedure mentioned above are executed to verifying the specificity of LAMP. The template was replaced by the genomic DNA extracted from *Leishmania donovani*, *Plasmodium vivax*, *Cryptosporidium parvum*, *Entamoeba histolytica*, *Trypanosoma evansi*. The amplified product was identified by 1.5% gel electrophoresis. LAMP-LFD was performed as a contrast.

### **Evaluation of sensitivity of LAMP-LFD**

The sensitivity of LAMP-LFD was evaluated against 10-fold serial dilutions of positive control template(genomic DNA of *T.gondii*) ranging from 1 ng to 0.01 fg, nuclease-free water were used as negative controls. Meanwhile, as comparative study, PCR was carried out under the protocol described

before. Outer forward primer (F3), outer backward primer (B3) of LAMP were used as a pair of upstream and downstream primers in PCR assay. The 25  $\mu$ l PCR reaction mixture was composed of 12.5  $\mu$ l 2  $\times$  Master Mix (Tsingke, Beijing, China), 5 pmol each of Forward and reverse primers, 2  $\mu$ l template. Amplification was performed at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 53 °C for 30 s, extension at 72 °C for 30 s, and a final extension step at 72 C for 10 min. The PCR products were visualized by 1.5% agarose gel electrophoresis stained with Gel-Red (Beyotime, Beijing, China).

### **Clinical application for detection of *T. gondii***

After the establishment of the universal rapid detection LAMP-LFD device for *T. gondii*, it was applied to the test of blood samples of stray animals (dogs, cats). One of 318 blood samples of dogs and cats collected from Zhejiang Province, China were extracted the genomic DNA. LAMP-LFD and PCR target 529 gene to detect *T. gondii* nucleic acids in blood samples.

## **Result**

### **The specificity of LAMP-LFD method.**

Genomic DNA samples of *Leishmania donovani*, *Plasmodium vivax*, *Cryptosporidium parvum*, *Entamoeba histolytica*, *Trypanosoma evansi* and *T. gondii* (RH) were used to test the specificity of LAMP and LAMP-LFD methods. Amplification signals were detected with the templates of *T. gondii* while the rest DNA templates showed no signals (Fig. 3A). LAMP-LFD was carried out using the templates above, positive bands at test lines were found when amplified with DNA samples of *T. gondii* and the rest remained negative (Fig. 3B), indicating that the established *T. gondii* LAMP-LFD detection method had good specificity.

### **Sensitivity of LAMP-LFD and PCR.**

Next, we performed the sensitivity analysis of LAMP-LFD using different concentrations of *T. gondii* genomic DNA as a template. The template concentration diluted by 10-fold from 1 ng to 0.01 fg was tested by real-time LAMP and LAMP-LFD. The signal curve of real-time LAMP shows that the detection limit of LAMP in 1 hour is 1 fg genomic DNA of *T. gondii* (Fig. 4A), same is LAMP-LFD (Fig. 4D). For conventional PCR assay, the minimum detectable concentration is found to be 100 fg (Fig. 4C).

### **Positive rate of *T. gondii* in stray dogs and cats.**

A total of 294 dogs and cats blood samples collected from five cities of Zhejiang province were detected by the established LAMP–LFD equipment, as well as the conventional PCR assay. As shown in Figure, positive detection rates were determined 4.72% (15/318) by LAMP-LFD (Fig. 6) and 0.63% (2/318) by conventional PCR assay.

Table 2.  
Total positive rate of *Toxoplasma gondii* in stray cats and dogs by LAMP-LFD method.

Sample	Positive (%)	Negative (%)	Total Number
Cat	5(4.76%)	81(95.24%)	105
Dog	10(4.69%)	198(95.31%)	213

## Discussion

Considering the global burden of toxoplasmosis, the unbearable consequences and the lack of effective anti-toxoplasmosis drugs, the development of early, rapid and cost-effective diagnosis methods suitable for economically deprived areas and field testing is essential for early screening, prevention, control, and treatment of toxoplasmosis. As LAMP is a molecular amplification technique characterized by high sensitivity and specificity that can amplify several copies of nucleic acid to  $10^9$  times. Thus, LAMP is gradually becoming an alternative to PCR methods in the molecular diagnosis of multiple pathogens. The key players in LAMP are the *Bst* DNA polymerase with strand displacement activity and a set of four primers recognizing six distinct sequences of the target fragment[20]. The application of LAMP in toxoplasmosis diagnosis has been widely reported. Burg *et al.* first proposed the B1 gene as a target gene for molecular diagnosis of toxoplasmosis, with 35 copies[33]; then the 529-bp fragment became another preferred target due to its up to 300 copies[34]. Therefore, we designed a set of optimal primers target 529 element in our study. The optimal system and reaction temperature for the LAMP reaction have been optimized in our previous work and therefore are not mentioned in this experiment[32]. Usually, the LAMP products can be identified by electrophoresis, turbidity measurement of magnesium pyrophosphate, fluorescent dye method, etc. Subsequently, to avoid the tediousness of electrophoretic, a faster and simpler method of chromatographic lateral flow dipstick (LFD) format was applied to reveal LAMP products[27, 35, 36]. LAMP-LFD is more specific for products detection attribute to it adopts molecular probe hybridization technique, biotin and fluorescein labeling of LAMP products in combination with double-sandwiching.

In this experiment, we applied the LAMP and LFD method for products detection. The biotin-labeled internal primers and FITC-labeled probes were used to make the ends of the stem-loop structure of the LAMP product biotin- and FITC-labeled, respectively, and the product was captured and visualized as it flowed through the anti-FITC antibody region of the lateral flow dipstick. This is more specific and sensitive than the magnesium pyrophosphate turbidity measurement and the fluorescent dye method. This LAMP-LFD method can detect down to 1 fg in 1 hour with template of genomic DNA of the *Toxoplasma gondii* RH strain and no across reaction with *Leishmania donovani*, *Plasmodium vivax*, *Cryptosporidium parvum*, *Entamoeba histolytica*, *Trypanosoma evansi*. Zhibing Lin *et al.* reported their detection limit was 10 fg with target 529 element by LAMP method[37]. Marco Lalle *et al.* propose that can detect *T. gondii* oocysts down to 25 oocysts/50 g in ready-to-eat baby lettuce with the LAMP-LFD assay[26]. Besides, Shirzad Fallahi *et al.* reported the detection limit of 529-LAMP was 1 fg *T. gondii*

DNA[38]. It proves that our LAMP-LFD method achieves equal detection potency with better specificity because only biotin- and FITC- amplicons resulting in a band detectable by the LFD strips.

Although infrequently reported, contamination by aerosol residues is an unavoidable obstacle in the field application for molecular diagnosis. LAMP amplification technology can amplify a large number of amplicons in a short time, thus greatly increasing the risk of aerosol contamination. Besides, the LAMP-LFD technique typically required opening the LAMP reaction tube for subsequent LFD testing, which greatly introduced the risk of aerosol contamination leading to false-positives result. Sterile pipetting technology and LAMP partition can be performed in the laboratory to reduce the risk of contamination[32]. Haihong Xu, Ming Hong *et al.* attempted to load the DNA fluorescent dye into a tin foil, microcrystalline wax-dye capsule and preloaded it into a LAMP reaction tube, which was centrifuged to mix the dye with the product and develop the color after the LAMP reaction was completed.[39, 40]. However, it has not yet been reported how to avoid contamination in LFD testing. In our study, we first report an integral hermetic LAMP-LFD device application for *T. gondii* detection. The operator only needs to prepare the LAMP reaction system in PCR tube according to the procedure, then connect the reaction tube to the device interface, and complete the LAMP amplification in a 65 °C water bath. The LFD assay is performed by turning the device upside down to mix the LAMP amplification product with the diluent, then laying the device flat to allow the mixture to flow onto the strip, and results can be visual read-out in the viewing window after 5 minutes. The whole testing process is carried out in the device, which greatly ensures the airtightness of the experiment and reduces the possibility of aerosol contamination. This device requires only a water bath for the LAMP reaction and is simple, cost-effective and suitable for use in minimally equipped laboratories and even field settings.

In this study, we detected 318 blood samples of stray cats and dogs collected from five cities in Zhejiang province for *Toxoplasma gondii* with LAMP-LFD. In view of the large variation in the number of *Toxoplasma gondii* in the animal blood on different stages of infection, in order to improve the nucleic acid extraction yield and detection rate, we divided each blood sample into three parts to extract nucleic acid and tested it with LAMP-LFD device. If anyone of them is positive, it means that the sample is positive. The total positive rate of *T. gondii* for stray cats and dogs are 4.76% and 4.69%, respectively, in our study. It is quite different from the seropositivity rate of *Toxoplasma gondii* in stray animals in the previous report. Mona K. Hegazy *et al.* compared the detection rates of *Toxoplasma gondii* in the blood of mice at different stages after infection with *Toxoplasma gondii* ME49 strain. On the seventh day after infection in mice, the LAMP method detected 18 positives out of the 20 examined samples. However, on day 56 post-infection, PCR and LAMP failed to detect positive from 20 mice blood samples. LAMP and routine PCR targeting the 529 bp RE gene failed to detect toxoplasmosis in the blood of mice in the chronic phase of the disease DNA, which can be explained by a progressive decrease in parasitemia levels as infection continues[18]. The application of LAMP for toxoplasma detection is therefore limited to the early stages of infection and is difficult to detect in peripheral blood at the quiescent bradyzoite stage. This explains the low detection rate of *Toxoplasma gondii* in the blood samples of our stray dogs and cats in Zhejiang province, which may be due to that the stray animals are already at an advanced stage of infection instead of parasitemia stage. Among them, the positive detection rate of

toxoplasmosis in cat blood samples was higher in Wenzhou. According to our field observations, these stray cats are in a more messy and unrestrained state and therefore more susceptible to toxoplasmosis infection. As the economy grew and the standard of living improved, some people started adopting stray animals from animal protection base as pets for companionship. However, pets such as dogs and cats as intermediate and final hosts of *T. gondii* increase the likelihood of human infection with *T. gondii* that supported by our prevalence rate investigation.

## Conclusion

In conclusion, we developed a novel *Toxoplasma gondii* detection assay, based on closed-device design test format that the LFD assay can be performed directly after the LAMP reaction completed. Our current results show that this LAMP-LFD device can be a reliable and portable diagnostic tool of *T. gondii*. The device has good airtightness, exhibits excellent sensitivity and specificity in sample testing and suitable for under-equipped laboratories and primary health care that will facilitate clinical diagnosis and epidemiological investigations for *T. gondii*.

## Declarations

### Ethics

This study was carried out in strict conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals according to the Animal Ethics Procedures and Guidelines of the Chinese National Institutes of Health. The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Zhejiang Academy of Medical Sciences (Approval ID: 2018-102).

### Acknowledgements and Funding

This work was supported by Provincial key R & D program of Zhejiang Department of Science and Technology (2019C03057), Health Commission of Zhejiang Province (2019PY025). We acknowledge Zhejiang Small Animal Protection Association for kindly providing the help in the process of collection of stray cats and dogs blood samples.

### Authors' contributions

QK and SL conceived of and designed the study. HD, JD and DL participated in the preparation of the lateral-flow-dipstick strips. QT and CX carried out the nucleic acid extraction of the sample. YX carried out the LAMP-LFD experiments and drafted the manuscript. XZ and BZ analyzed the data. HL revised the manuscript. All authors read and approved the final manuscript.

### Availability of data and materials

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

## Competing interests

The authors declare that they have no competing interests.

## Consent for publication

Not applicable.

## References

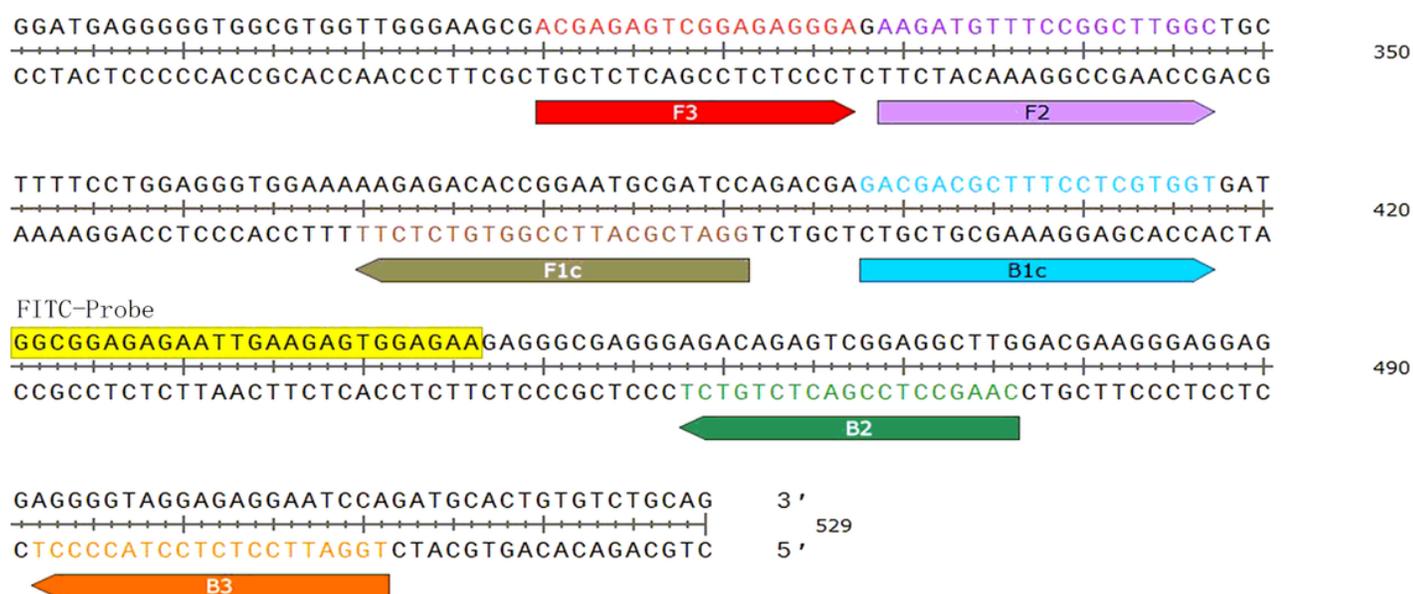
1. Blume M, Seeber F. Metabolic interactions between *Toxoplasma gondii* and its host. *F1000Res*. 2018;7; doi: 10.12688/f1000research.16021.1.
2. Jones JL, Dubey JP. Foodborne toxoplasmosis. *Clin Infect Dis*. 2012;55 6:845-51; doi: 10.1093/cid/cis508.
3. Hill D, Dubey JP. *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clin Microbiol Infect*. 2002;8 10:634-40; doi: 10.1046/j.1469-0691.2002.00485.x.
4. Dong H, Su R, Lu Y, Wang M, Liu J, Jian F, et al. Prevalence, Risk Factors, and Genotypes of *Toxoplasma gondii* in Food Animals and Humans (2000-2017) From China. *Front Microbiol*. 2018;9:2108; doi: 10.3389/fmicb.2018.02108.
5. Li K, Wang M, Zhang H, Lei Z, Zhang L, Luo H, et al. Epidemiology of *Toxoplasma gondii* infection in native Tibetans in Tibet, China. *Acta Parasitol*. 2017;62 3:529-32; doi: 10.1515/ap-2017-0063.
6. Augusto L, Amin PH, Wek RC, Sullivan WJ, Jr. Regulation of arginine transport by GCN2 eIF2 kinase is important for replication of the intracellular parasite *Toxoplasma gondii*. *PLoS Pathog*. 2019;15 6:e1007746; doi: 10.1371/journal.ppat.1007746.
7. Berrouch S, Escotte-Binet S, Harrak R, Huguenin A, Flori P, Favennec L, et al. Detection methods and prevalence of transmission stages of *Toxoplasma gondii*, *Giardia duodenalis* and *Cryptosporidium* spp. in fresh vegetables: a review. *Parasitology*. 2020;147 5:516-32; doi: 10.1017/S0031182020000086.
8. De Berardinis A, Paludi D, Pennisi L, Vergara A. *Toxoplasma gondii*, a Foodborne Pathogen in the Swine Production Chain from a European Perspective. *Foodborne Pathog Dis*. 2017;14 11:637-48; doi: 10.1089/fpd.2017.2305.
9. Zhou Y, Zhang H, Cao J, Gong H, Zhou J. Epidemiology of toxoplasmosis: role of the tick *Haemaphysalis longicornis*. *Infect Dis Poverty*. 2016;5:14; doi: 10.1186/s40249-016-0106-0.
10. Skotarczak BI. The role of ticks in transmission cycle of *Toxoplasma gondii*. *Ann Parasitol*. 2016;62 3:185-91; doi: 10.17420/ap6203.52.
11. Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet*. 2004;363 9425:1965-76.
12. Wang Z-D, Liu H-H, Ma Z-X, Ma H-Y, Li Z-Y, Yang Z-B, et al. Infection in Immunocompromised Patients: A Systematic Review and Meta-Analysis. *Frontiers in microbiology*. 2017;8:389; doi: 10.3389/fmicb.2017.00389.

13. Fuglewicz AJ, Piotrowski P, Stodolak A. Relationship between toxoplasmosis and schizophrenia: A review. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University*. 2017;26 6:1031-6; doi: 10.17219/acem/61435.
14. Bigna JJ, Tochie JN, Tounouga DN, Bekolo AO, Ymele NS, Simé PS, et al. Global, regional and national estimates of seroprevalence in pregnant women: a protocol for a systematic review and modelling analysis. *BMJ open*. 2019;9 10:e030472; doi: 10.1136/bmjopen-2019-030472.
15. Dunay IR, Gajurel K, Dhakal R, Liesenfeld O, Montoya JG. Treatment of Toxoplasmosis: Historical Perspective, Animal Models, and Current Clinical Practice. *Clin Microbiol Rev*. 2018;31 4; doi: 10.1128/CMR.00057-17.
16. Kodym P, Malý M, Beran O, Jilich D, Rozsypal H, Machala L, et al. Incidence, immunological and clinical characteristics of reactivation of latent *Toxoplasma gondii* infection in HIV-infected patients. *Epidemiol Infect*. 2015;143 3:600-7; doi: 10.1017/s0950268814001253.
17. Ji-Long S, Li Y. [Prevalence and fundamental researches of prevention and treatment of toxoplasmosis in China: an overview]. *Zhongguo Xue Xi Chong Bing Fang Zhi Za Zhi*. 2019;31 1:71-6; doi: 10.16250/j.32.1374.2019012.
18. Hegazy MK, Awad SI, Saleh NE, Hegazy MM. Loop mediated isothermal amplification (LAMP) of *Toxoplasma* DNA from dried blood spots. *Exp Parasitol*. 2020;211:107869; doi: 10.1016/j.exppara.2020.107869.
19. Rostami A, Karanis P, Fallahi S. Advances in serological, imaging techniques and molecular diagnosis of *Toxoplasma gondii* infection. *Infection*. 2018;46 3:303-15; doi: 10.1007/s15010-017-1111-3.
20. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. 2000;28 12:E63.
21. Tomita N, Mori Y, Kanda H, Notomi T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature protocols*. 2008;3 5:877-82; doi: 10.1038/nprot.2008.57.
22. Aonuma H, Yoshimura A, Kobayashi T, Okado K, Badolo A, Nelson B, et al. A single fluorescence-based LAMP reaction for identifying multiple parasites in mosquitoes. *Exp Parasitol*. 2010;125 2:179-83; doi: 10.1016/j.exppara.2009.12.023.
23. Ravan H, Yazdanparast R. Development and evaluation of a loop-mediated isothermal amplification method in conjunction with an enzyme-linked immunosorbent assay for specific detection of *Salmonella* serogroup D. *Anal Chim Acta*. 2012;733:64-70; doi: 10.1016/j.aca.2012.04.034.
24. Nimitphak T, Kiatpathomchai W, Flegel TW. Shrimp hepatopancreatic parvovirus detection by combining loop-mediated isothermal amplification with a lateral flow dipstick. *J Virol Methods*. 2008;154 1-2:56-60; doi: 10.1016/j.jviromet.2008.09.003.
25. Zhang J, Cao J, Zhu M, Xu M, Shi F. Loop-mediated isothermal amplification-lateral-flow dipstick (LAMP-LFD) to detect *Mycoplasma ovipneumoniae*. *World J Microbiol Biotechnol*. 2019;35 2:31; doi: 10.1007/s11274-019-2601-5.

26. Lalle M, Possenti A, Dubey JP, Pozio E. Loop-Mediated Isothermal Amplification-Lateral-Flow Dipstick (LAMP-LFD) to detect *Toxoplasma gondii* oocyst in ready-to-eat salad. *Food Microbiol.* 2018;70:137-42; doi: 10.1016/j.fm.2017.10.001.
27. Yang Y, Li Q, Wang S, Chen X, Du A. Rapid and sensitive detection of *Babesia bovis* and *Babesia bigemina* by loop-mediated isothermal amplification combined with a lateral flow dipstick. *Veterinary parasitology.* 2016;219:71-6; doi: 10.1016/j.vetpar.2016.02.004.
28. Sun YL, Yen CH, Tu CF. Visual detection of canine parvovirus based on loop-mediated isothermal amplification combined with enzyme-linked immunosorbent assay and with lateral flow dipstick. *J Vet Med Sci.* 2014;76 4:509-16; doi: 10.1292/jvms.13-0448.
29. Njiru ZK. Rapid and sensitive detection of human African trypanosomiasis by loop-mediated isothermal amplification combined with a lateral-flow dipstick. *Diagn Microbiol Infect Dis.* 2011;69 2:205-9; doi: 10.1016/j.diagmicrobio.2010.08.026.
30. Valian HK, Mirhendi H, Mohebbali M, Shojaee S, Fallahi S, Jafari R, et al. Comparison of the RE-529 sequence and B1 gene for *Toxoplasma gondii* detection in blood samples of the at-risk seropositive cases using uracil DNA glycosylase supplemented loop-mediated isothermal amplification (UDG-LAMP) assay. *Microb Pathog.* 2020;140:103938; doi: 10.1016/j.micpath.2019.103938.
31. Tong Q, Chen R, Kong Q, Goossens J, Radwanska M, Lou D, et al. DNA detection of *Trypanosoma evansi*: Diagnostic validity of a new assay based on loop-mediated isothermal amplification (LAMP). *Veterinary parasitology.* 2018;250:1-6; doi: 10.1016/j.vetpar.2017.12.006.
32. Kong QM, Lu SH, Tong QB, Lou D, Chen R, Zheng B, et al. Loop-mediated isothermal amplification (LAMP): early detection of *Toxoplasma gondii* infection in mice. *Parasit Vectors.* 2012;5:2; doi: 10.1186/1756-3305-5-2.
33. Burg JL, Grover CM, Pouletty P, Boothroyd JC. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J Clin Microbiol.* 1989;27 8:1787-92.
34. Homan WL, Vercammen M, De Braekeleer J, Verschueren H. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. *International journal for parasitology.* 2000;30 1:69-75; doi: 10.1016/s0020-7519(99)00170-8.
35. Kiatpathomchai W, Jaroenram W, Arunrut N, Jitrapakdee S, Flegel TW. Shrimp Taura syndrome virus detection by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. *J Virol Methods.* 2008;153 2:214-7; doi: 10.1016/j.jviromet.2008.06.025.
36. Yongkiettrakul S, Jaroenram W, Arunrut N, Chareanchim W, Pannengetch S, Suebsing R, et al. Application of loop-mediated isothermal amplification assay combined with lateral flow dipstick for detection of *Plasmodium falciparum* and *Plasmodium vivax*. *Parasitol Int.* 2014;63 6:777-84; doi: 10.1016/j.parint.2014.06.004.
37. Lin Z, Zhang Y, Zhang H, Zhou Y, Cao J, Zhou J. Comparison of loop-mediated isothermal amplification (LAMP) and real-time PCR method targeting a 529-bp repeat element for diagnosis of toxoplasmosis. *Veterinary parasitology.* 2012;185 2-4:296-300; doi: 10.1016/j.vetpar.2011.10.016.

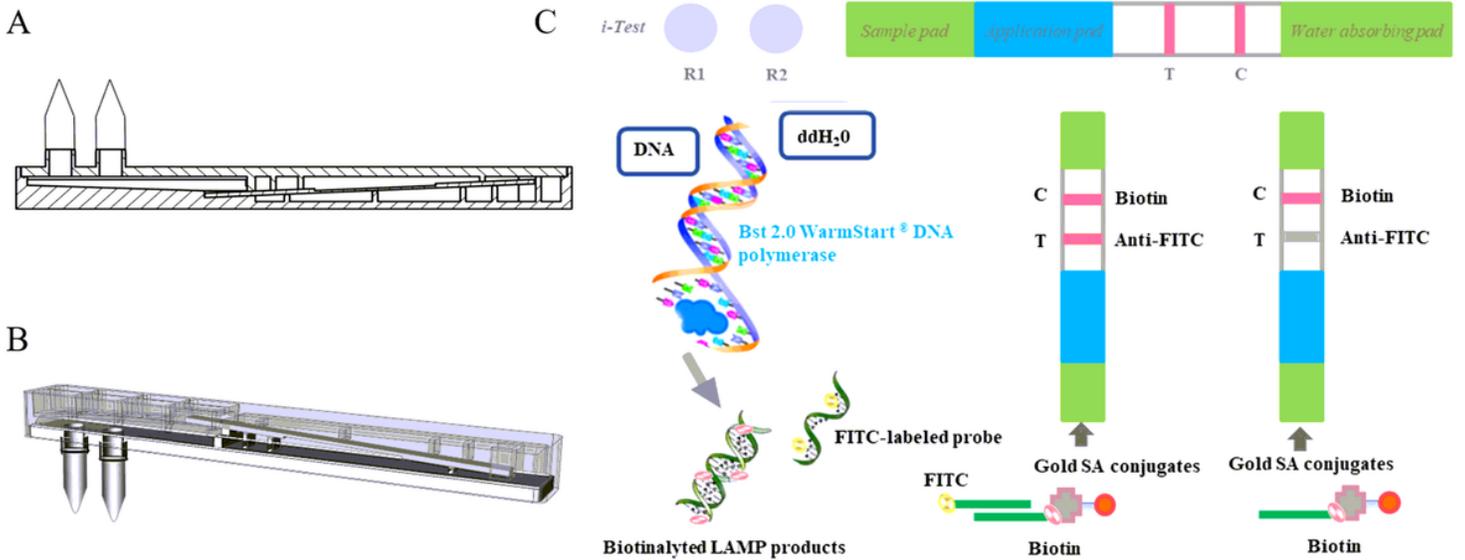
38. Fallahi S, Mazar ZA, Ghasemian M, Haghighi A. Challenging loop-mediated isothermal amplification (LAMP) technique for molecular detection of *Toxoplasma gondii*. *Asian Pac J Trop Med*. 2015;8 5:366-72; doi: 10.1016/S1995-7645(14)60345-X.
39. Hong M, Zha L, Fu W, Zou M, Li W, Xu D. A modified visual loop-mediated isothermal amplification method for diagnosis and differentiation of main pathogens from *Mycobacterium tuberculosis* complex. *World J Microbiol Biotechnol*. 2012;28 2:523-31; doi: 10.1007/s11274-011-0843-y.
40. Xu H, Zhang L, Shen G, Feng C, Wang X, Yan J, et al. Establishment of a novel one-step reverse transcription loop-mediated isothermal amplification assay for rapid identification of RNA from the severe fever with thrombocytopenia syndrome virus. *J Virol Methods*. 2013;194 1-2:21-5; doi: 10.1016/j.jviromet.2013.07.037.

## Figures



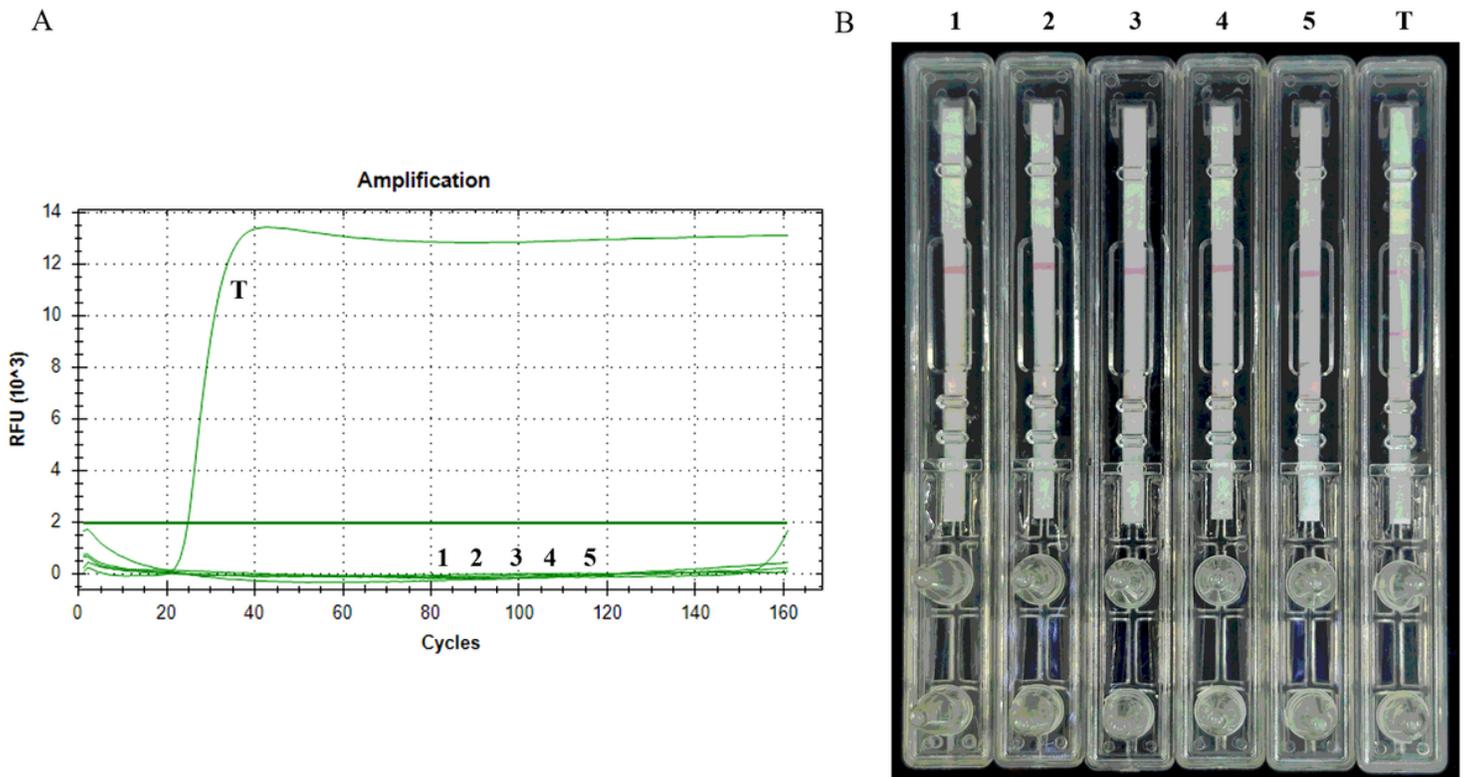
**Figure 1**

The nucleotide sequence of 529 showing a set of primers and the probe. The sequence with color red, purple, brown, blue, green, orange represent primer F3, F2, F1c, B1c, B2, B3 respectively. The forward inner primer (FIP = F1c-F2) was labeled with biotin in the in the 5' end and the primer amplify from 5'end to 3'end. The yellow module shows the sequence of FITC-probe between primer B1c and B2.



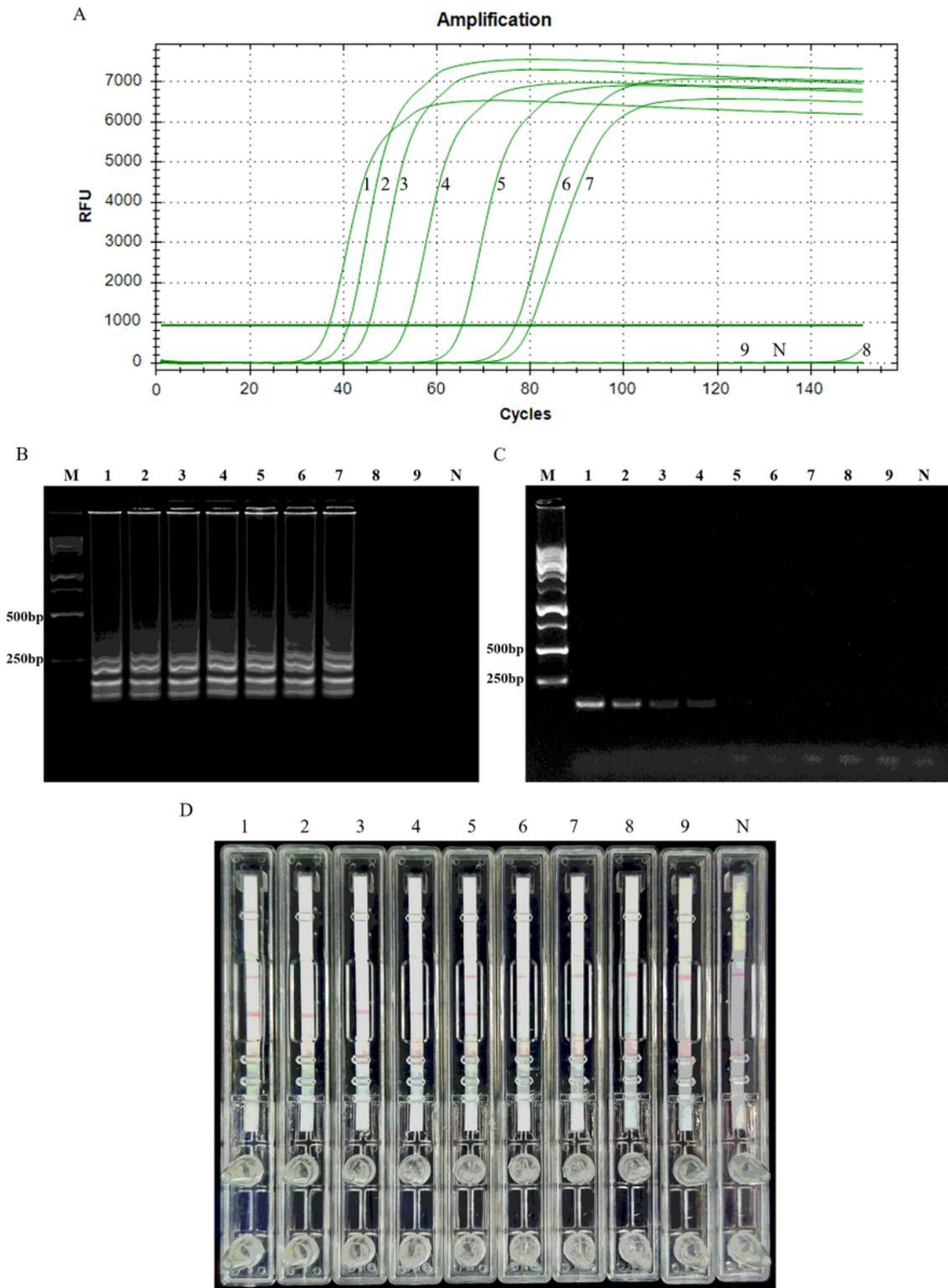
**Figure 2**

The LAMP-LFD device and principle. A. Side view of LAMP-LFD equipment. B. Schematic diagram of LAMP-LFD model. C. Schematic of the working principle of LAMP-LFD.



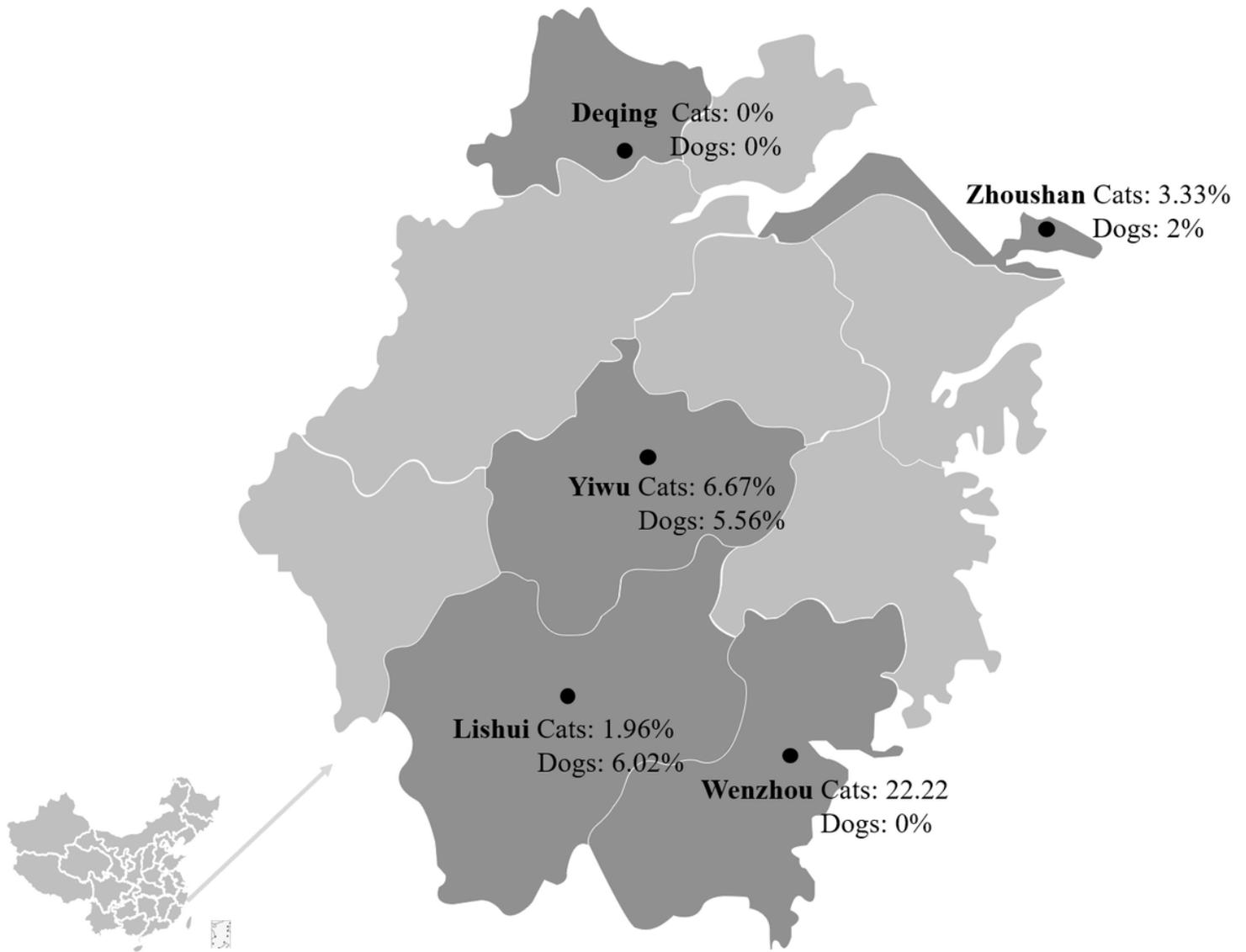
**Figure 3**

The results for specificity of LAMP and LAMP-LFD detection. (A) The curves of real-time LAMP. (B) Visual inspection of LAMP-LFD. (1) *Leishmania donovani* (2) *Plasmodium vivax* (3) *Cryptosporidium parvum* (4) *Entamoeba histolytica* (5) *Trypanosoma evansi* (T) *T. gondii* (RH).



**Figure 4**

Comparative results of Sensitivity for LAMP-LFD and PCR. (A) The signal curve of real-time LAMP. (B) Agarose gel electrophoresis of the *Toxoplasma gondii* LAMP products. (C) Agarose gel electrophoresis of the *Toxoplasma gondii* PCR products. (D) Visual inspection of LAMP-LFD. Lane M, DNA ladder marker; Number 1–8 represents 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg, 0.01 fg of *Toxoplasma gondii* DNA, respectively; lane N, negative control.



**Figure 5**

Positive rate of *Toxoplasma gondii* in stray cats and dogs in five cities of Zhejiang Province. Cats and Dogs represent positivity rates for cats and dogs, respectively. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.