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A novel dynamic flow immunochromatographic test (DFICT) using gold nanoparticles for the serological detection of *Toxoplasma gondii* infection in dogs and cats

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ABSTRACT

A novel dynamic flow immunochromatographic test (DFICT) is proposed for rapid assay utilizing *Toxoplasma gondii* as a model. The test is based on a proprietary technology that combines the principles of immunochromatography and fluid dynamics. Gold nanoparticles conjugated to staphylococcal protein A (SPA) were prepared in liquid form and used as signal vehicles. *T. gondii*-specific recombinant antigens and SPA were sprayed onto a nitrocellulose membrane in strips at positions designated as T and C, respectively. The DFICT is performed by applying a 100 μL aliquot of liquid gold-SPA conjugate to the reagent hole and a 5 μL aliquot of serum sample to the sample hole. The results were observable within 5 min by the naked eye. The lowest detectable limit of the assay was determined as the highest dilution (1:320) of positive serum. No cross-reaction of the antibodies with other related canine or feline pathogens was observed. The DFICT can be stored for 12 months at 4 °C or 6 months with no loss of sensitivity or specificity. A high degree of consistency was observed between the DFICT and the standard ELISA kit, supporting the reliability of the novel test strip. The introduction of a liquid gold nanoparticle conjugate reagent provides this method with several attractive characteristics, such as ease of manufacture, low sample volume requirements, high selectivity and high efficiency. This method opens a novel pathway for rapid diagnostic screening and field analysis.

1. Introduction

Toxoplasmosis is a worldwide endemic disease caused by *Toxoplasma gondii*, a parasite that infects a broad spectrum of vertebrate hosts, including humans (Cook et al., 2000; Commodaro et al., 2009; Tenter et al., 2000). Both cats and dogs infected by *T. gondii* pose a potential threat to public health (Dubey et al., 2009; Hill, Dubey, 2002; Lindsay et al., 1997; Stagno et al., 1980). Thus, it is important to evaluate the role of domestic animals in *T. gondii* transmission. The development of sensitive and specific methods for the detection of *T. gondii* infection is a key step toward treating and managing patients with suspected toxoplasmosis. Serological techniques play a major role in the diagnosis of toxoplasmosis in humans and animals. Several serologic methods have been standardized for the detection of *T. gondii* infection. Among these methods, the indirect hemagglutination test, the latex agglutination test, the indirect fluorescent antibody test, and enzyme-linked immunosorbent assay (ELISA) are the most common (György et al., 2011; Li et al., 2000; Montoya, 2002; Remington et al., 2004; Wang et al., 2011;Wang et al., 2012). However, these procedures are time consuming, require expensive equipment and well-trained personnel, and can only be used in laboratories.

The immunochromatographic test (ICT) has become a well-established and accepted point-of-care testing technique. The most widely used format for such assays uses gold nanoparticles for colorimetric detection (Meng et al., 2014; Nakayama et al., 2014). The antibodies bind tightly to the surfaces of the gold nanoparticles when correctly coupled, providing long-term stability in liquid and solid forms (Xie et al., 2014). Many studies have reported the successful detection of pathogens using this assay (Pengsuk et al., 2013; Sun et al., 2013). Some studies also have focused on detection of antibodies against *T. gondii* using this assay (Terkawi et al., 2013). Compared with other serologic tests, ICT is an economical, simple, and rapid approach, which makes it suitable for clinical and field applications (Peng et al., 2007).

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However, considerable efforts still need to be made to improve ICT in the areas of manufacturing processes, sample volume requirements and production costs.

Herein, we developed a novel DFICT based on a proprietary technology that combines the principles of immunochromatography and fluid dynamics. The advantages of using the liquid gold conjugate reagent in this novel test include ease of manufacture, low production cost, low sample volume requirements, high selectivity and high efficiency. In the current study, *T. gondii* was used as a model analyte to demonstrate the use of this new method. The accuracy of the DFICT was further compared with that of a standard ELISA kit using clinical serum samples collected in the field.

2. Materials and methods

2.1. Serum samples and materials

Two standard positive dog sera for *T. gondii* were obtained from experimentally infected dogs in our laboratory. Two standard positive cat control sera for *T. gondii* were provided from experimentally infected cats by Dr. Zhou Peng (Chinese Academy of Agricultural Sciences, Shanghai Veterinary Research Institute). Furthermore, twenty-five additional positive dog sera and fifteen positive cat sera were obtained from animals naturally infected with *T. gondii* in our previous study (Wang et al., 2011, 2012). All of the thirty standard negative serum samples from healthy dogs and cats were obtained from stocks that have been preserved in our laboratory. Positive dog serum controls against different non-*T. gondii* pathogens, including canine distemper virus (CDV), canine parvovirus (CPV), canine coronavirus (CCoV), canine leishmania (CanL), and *Neospora caninum* (*N.caninum*), were obtained from our laboratory stocks. Positive cat serum controls against different non-*T. gondii* pathogens for feline panleukopenia virus (FPV), feline calicivirus (FCV) and *N.caninum* were also obtained from our laboratory stocks. Glass fiber membranes, NC membranes, absorbent pads, and PVC sheets were purchased from Millipore Corporation (Shanghai, China). Hydrogen tetrachloroaurate hydrate ($HAuCl_4$), trisodium citrate, bovine serum albumin (BSA), and SPA were purchased from Sigma Chemical Company (USA). Approximately 0.02 M sodium phosphate-buffered saline (PBS; pH 8.5) was used as a serum dilution buffer. All solvents, chemicals, and salts used in this study were of analytical grade. Solutions were prepared using Milli-Q18 Ω water (Millipore Purification System).

2.2. Preparation of immunoassay reagents

Several studies have shown that surface antigens (SAGs) are highly conserved in isolated *T. gondii* strains and, therefore, qualify as potential candidates for diagnosis of the parasite (Pietkiewicz et al., 2004; Kotresha and Noordin, 2010). The preparations of recombinant proteins (SAG1 and SAG2) are described in our previous studies (An et al., 2009; Nie et al., 2010). Briefly, two recombinant expression plasmids, pET32a-tSAG1 and pET32a-tSAG2 (kept in our laboratory), were transformed into *Escherichia coli* BL21 (DE3)-competent cells. After expression and purification, the two *T. gondii*-specific recombinant proteins were used as capture reagents fixed on the NC membrane (test line).

2.3. Preparation of gold-SPA conjugates

Colloidal gold particles were prepared as described previously (Meng et al., 2014), with modifications. Briefly, 100 mL 0.01% $HAuCl_4$ solution was boiled for 2 min, and 3 mL 1% trisodium citrate solution (w/v) was added under constant stirring. As the solution cooled to room temperature (RT), the pH was adjusted to 6.5 with 0.2 M K_2CO_3. Approximately 1 mL SPA (1.0 mg mL^{-1}) was mixed gently and with constant stirring with a 100 mL colloidal gold solution prepared above and incubated for 30 min at RT. BSA was added to a final concentration of 1% (w/v) to stabilize and block conjugate particles. After centrifugation, the conjugate pellet was suspended in 0.01 M PBS containing 1% (w/v) BSA, 0.3% (v/v) Tween-20, 0.9% (w/v) NaCl, and 0.05% (w/v) sodium azide and stored at 4 °C. The colloidal gold particles, either unconjugated or conjugated to SPA, were characterized by transmission electron microscopy (TEM) and UV–vis spectrophotometer. The liquid secondary antibody conjugate reagent was packed in small bottles until use.

2.4. Preparation of the DFICT strip

The DFICT comprises a test strip including an NC membrane, a glass fiber membrane, and an absorbent pad. It is not necessary to prepare the conjugated pad and the sample pad as the conventional colloidal gold-based test strip. Images of the ready-to-use strip test and opened cassette are presented in Fig. S1. Briefly, a mixture of the recombinant proteins (SAG1: 1 mg mL^{-1}; SAG2: 1 mg mL^{-1}) and SPA (1 mg mL^{-1}) was used as a capture reagent separately microsprayed at 1 μl cm^{-1} onto the test (marked "T") and control lines (marked "C") situated 4 mm apart in the middle of the NC membrane (25 mm × 300 mm; Millipore, Bedford, MA, USA) using Quanti 3000 Biojets attached to a XYZ Biostrip Dispenser (Bio-Dot, Irvine, CA, USA). The nominal capillary flow time and thickness of the membrane were 140 s/cm and 135 μm, respectively. After drying for 3 h at 37 °C, the membrane was sealed in a plastic bag and stored under dry conditions at RT.

The absorbent pad, which was made from 100% pure cellulose fiber, was cut into 20 mm × 300 mm sections. The glass fiber membrane cut into 20 mm × 200 mm sections was added to the liquid gold conjugate. The blotting NC membrane, glass fiber membrane, and absorbent pad were assembled sequentially on a plastic-backed support card with 1–2 mm overlap of each component; one end of the glass fiber membrane overlapped with the blotting membrane, and the other end of the blotting membrane was attached to the absorption pad to remove excess reaction mixture. This assembly was cut into 4 mm wide strips using a CM-4000 Cutter (Bio-Dot, Irvine, CA, USA). The liquid gold conjugate reagent was stored in a small bottle at 4 °C. Strips were housed in a plastic cassette with silica desiccant gel and stored under dry conditions at RT until use.

2.5. Specificity, sensitivity, and stability of the DFICT

The negative dog and cat serum samples from healthy animals and the positive samples against different non-*T.gondii* pathogens, including *N. caninum*, CDV, CPV, CCoV, CanL, FPV and FCV, were used to evaluate the specificity of the DFICT. Standard positive dog and cat serum samples for *T. gondii* were used as positive controls. An aliquot (5 μL) of serum sample was added for testing, and 0.01 M PBS (pH 7.2) was used as the blank control. Each sample was tested in triplicate with the strip assay. The sensitivity of the developed strip was also determined with serial dilutions of standard positive dog and cat sera against *T. gondii*. Positive sera were diluted with 0.01 M PBS in a series (1:2 to 1:1280), and the procedure was repeated more than three times. PBS (0.01 M) was used as the blank control. Two standard positive dog and cat sera for *T. gondii* were used as positive controls.

To establish the stability of the DFICT, several of the conjugated reagents were stored for 3, 6, 9, and 12 months at RT and 4 °C. The stored strips were re-examined for specificity, sensitivity, and appearance with known *T. gondii*-positive and *T. gondii*-negative sera.

2.6. Comparison of the DFICT with a reference ELISA

The sensitivity and specificity of the DFICT were verified with 40 positive serum samples and 36 negative serum samples preserved in our laboratory or obtained from our previous study (Wang et al., 2011, 2012). The same sets of samples were also tested in parallel using a commercially available ID Screen® Toxoplasmosis indirect multi-species ELISA kit (IDVET, France) based on the P30 antigen (SAG1).

Furthermore, we performed clinical evaluations of the DFICT as an antibody detection tool in 241 dog and 97 cat sera collected randomly from animals presented for annual vaccination to the animal health authorities and pet hospitals in Shanghai and Nantong from 2013 to 2014. Separated sera were stored at −20 °C until analysis. Each sample was tested in triplicate using three different batches of the DFICT strips. Clinical serum samples were additionally examined for antibodies against *T. gondii* using the reference ELISA. Positive and negative control sera were provided in the kit. The procedure was performed according to the manufacturer's recommendations. All values were recorded after the appropriate blank correction. ELISA results were considered positive when the value of a 10 μL serum sample was 2.1 times higher than that of the negative control at an optical density of 450 nm. DFICT samples were considered positive when both control and test lines turned purplish-red upon application of 5 μL serum.

3. Results and discussion

3.1. Preparation of gold-SPA conjugates

Gold nanoparticles were synthesized via chemical condensation, whereby $HAuCl_4$ was reduced to neutral gold atoms with sodium citrate. TEM images revealed that the gold particles were uniform with an average size of 15 nm (Fig. 1A), and the gold particles were found to be stable after conjugation (Fig. S2). Along with the SPA adsorbed on the surface of gold nanoparticles, its size increased and the absorb peak shifted. The UV–vis spectra of the gold particles displayed maximum absorbance at 518 nm, while the maximum absorbance of the gold-SPA conjugate occurred at a wavelength of 524 nm (Fig. 1B).

3.2. Detection procedure and principles of the DFICT

As shown in Fig. 2A, detection was initially performed by placing 100 μL (approximately 2 full drops) of gold-SPA conjugate reagents into the reagent hole (marked "R"). The gold-conjugated SPA migrated across the NC membrane and passed through the sample hole, as indicated by the red liquid front. An aliquot (5 μL) of the serum sample was added to the sample hole (marked "S"), which migrated across the membrane. The principle of the DFICT is illustrated in Fig. 2B. The assay is based on an indirect immunoassay format. Specific anti-*Toxoplasma* antibodies (IgG) in positive serum reacted with the gold-SPA conjugate to form a gold-SPA-antibody complex, which was captured by *T. gondii*-specific recombinant antigens on the NC membrane to form a gold-SPA-antibody-antigen complex that generated a red band on the test line (marked "T"). The density of the red band was proportional to the concentration of *T. gondii* antibody used. Excess gold-SPA conjugate reacted with nonspecific antibody (IgG) in the serum sample, flowed over the test line, and bound purified SPA on the control line (marked "C"), forming another red band at the control line of the strip. The results could be assessed with the naked eye within 5 min. After migration of the mixture through the membrane at RT, negative results were judged by the appearance of a single line in C. In cases where no line was evident at the control position, the test was considered invalid.

In many conventional ICT assays, the procedure calls for dispensing a serum sample followed by chasing with a buffer to drive the liquid forward (Meng et al., 2014; Shen et al., 2010). The DFICT does not require washing or blocking buffers during sample addition, between the sample and visualization reagent addition steps, or at the termination of the reaction. Furthermore, in many conventional ICT assays, the liquid conjugated gold needs to be impregnated on glass fiber membranes to generate the conjugated pad and subsequently dried in a vacuum or dryer room. In DFICT, the use of the liquid gold conjugate reagent instead of dried conjugate makes the manufacturing process simple, as no drying facilities or low humidity control are required for device assembly. In addition, individual air-tight packing of the assembled device is not required. The reason for having a glass pad in the strip is merely to accommodate the applied liquid gold when the strip is placed in a lateral position (out of convenience). If the strip were vertically oriented and placed in a tube containing the liquid gold, there would be no need for the glass pad.

Furthermore, by using the liquid gold conjugate as the reporter

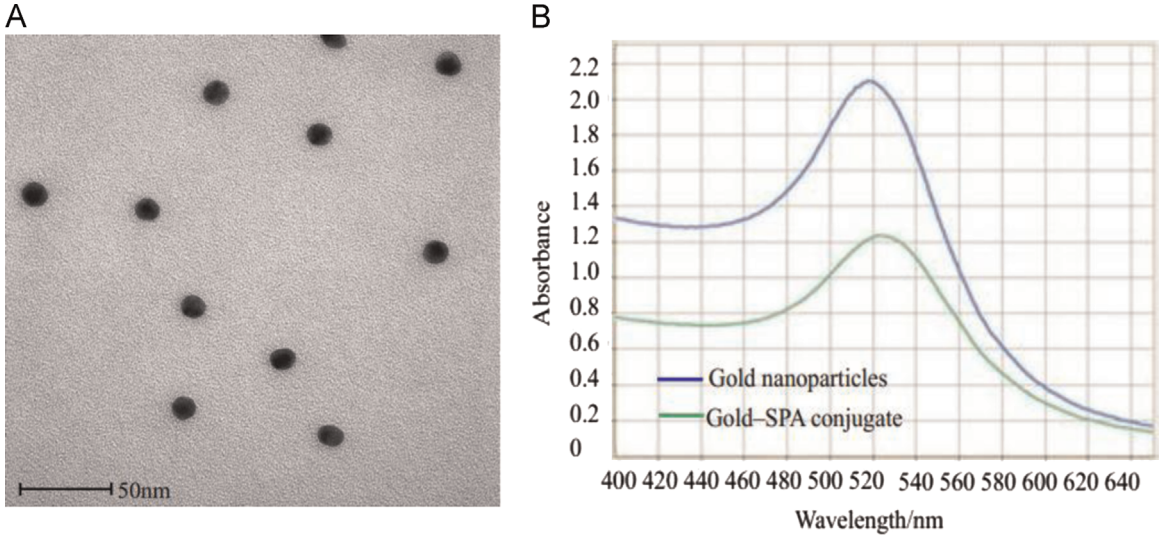

Fig. 1. (A) TEM images of gold nanoparticles. (B) UV–vis spectra of gold nanoparticles solution and gold-SPA conjugate solution.

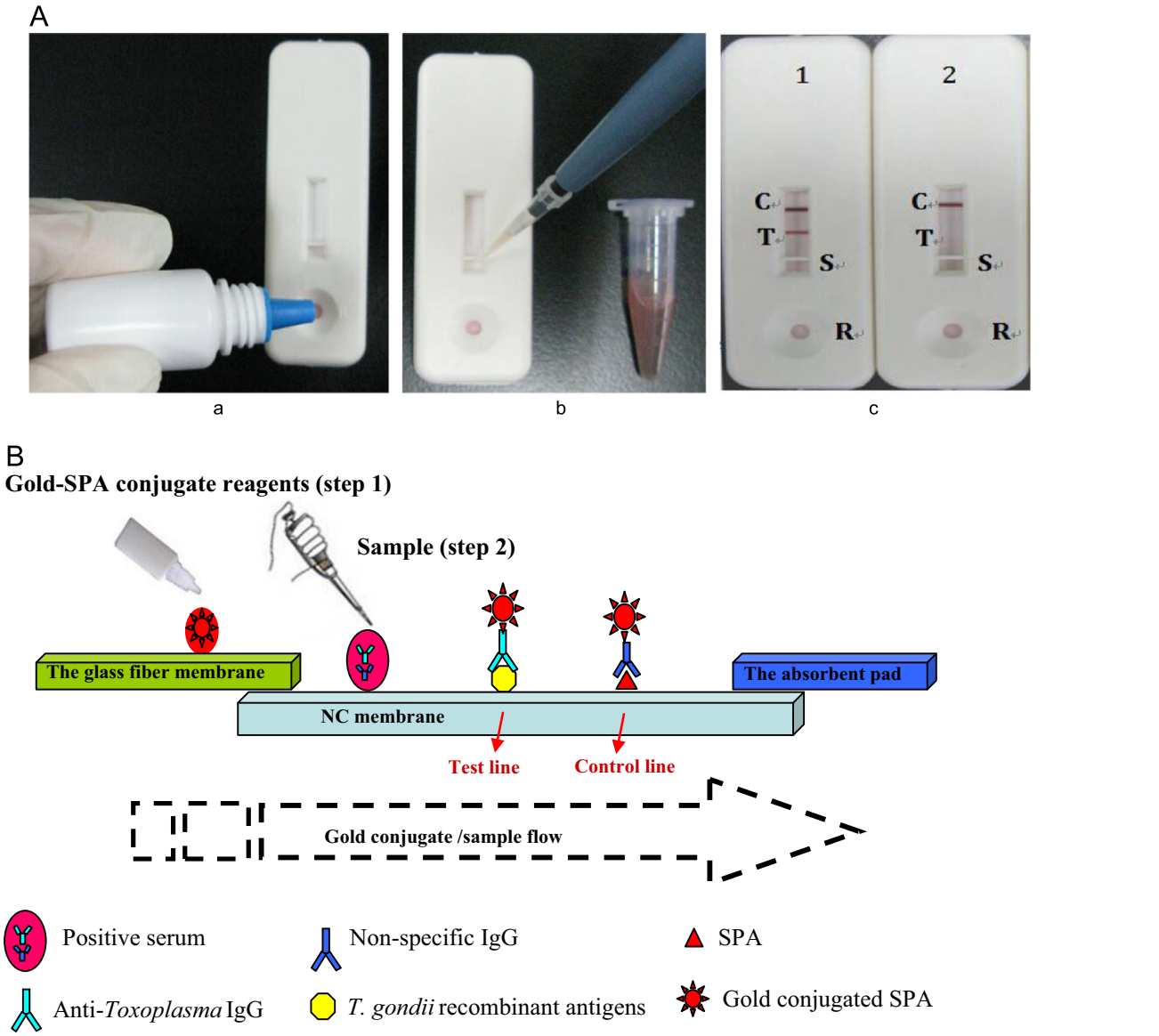

Fig. 2. A. Steps involved in DFICT for naked-eye sensitive detection: (a) adding 2 full drops of a liquid secondary antibody conjugate; (b) adding 5 μL of a serum sample; (c) results determination: 1, positive result; 2, negative result. B. Schematic illustration of DFICT. The gold conjugated SPA reagent was added on the glass fiber membrane. Following the application of a sample containing specific anti-*Toxoplasma* antibodies on the NC membrane, the conjugated antibody and antigen complex were captured by the *T. gondii*-specific recombinant antigens on the test line (T), resulting in a reddish purple band of gold nanoparticle at position T. The unbound conjugated antibody moved across the test line and was captured by purified SPA (red triangles) to form a band at position C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reagent, the DFICT ensures that a sufficient and constant supply of the gold conjugate reagent can be applied with only a small volume of serum (i.e., a 5 μL serum sample) compared to conventional ICT. This set up facilitates migration to the test area (T line) for reaction with captured antigen with a minimum loss of specific antibodies. The result is the rapid arrival of the sample at the reaction zone and maximum antibody availability to react with the capture antigen, thus achieving high sensitivity. In addition, the gold conjugate reagent is not exhausted in binding to extraneous nonspecific antibodies left by the serum on the path to the control zone (C line) and no Hook effects can occur.

In the conventional ICT assay, the volume of serum sample needed is usually as high as 50 or 100 μL (Meng et al., 2014; Shen et al., 2010), and a sample pad is often needed to accommodate the serum sample, which will then diffuse into the conjugate pad. Furthermore, to reduce or counteract matrix interference throughout the reaction system, the sample pad needs to be pretreated or the serum sample is mixed with a serum dilution buffer before being added to the sample hole (Pengsuk et al., 2013; Wang et al., 2011; Terkawi et al., 2013). In our new method, only a 5 μL serum sample is needed, and the sample can be applied directly onto the membrane without serum dilution buffer, making the sample pad unnecessary.

The DFICT is performed in a sequential manner with the addition of the liquid gold conjugate followed by the addition of a serum sample. However, whether we add the sample onto the membrane first followed by the addition of liquid gold or reverse the sequence, the ultimate result is the same. The chief difference is that when the test is performed as first described, and not in reverse, the result appears faster and the background works better for very viscous samples. As such, if a user performs the test in a reverse sequence, there is no danger of obtaining a spurious result.

More interestingly, the test capture reagent SPA has the capacity to bind to the Fc fragment of the IgG of human and other

mammals without interacting with the antigen-binding site (Zhang et al., 2006). Theoretically, the DFICT strip is therefore suitable for the diagnosis of toxoplasmosis in humans and other mammals, which we plan to test in the future. In addition, the use of the same conjugate reagent as a "sample carrier" and "reporter" makes the reagent system simple to manufacture and easy to use in the field. Moreover, the same bottle of gold-SPA conjugate reagent can be used to detect other pathogens, simply requiring a change of the specific antigens for the test line on the NC membrane.

3.3. Specificity of the DFICT

The dog and cat sera from healthy animals were all found to be negative for anti-*T.gondii* antibodies with the DFICT. Furthermore, both dog and cat anti-*T. gondii* standard serum samples were tested positive with this assay, as indicated by the appearance of two visible red bands at the test and control line positions. The other positive serum samples against non-*T.gondii* pathogens were confirmed based on the presence of a single red band at the control line (Fig. 3). Similar result patterns were reproduced in repeated experiments. Our results indicated a high specificity of this method for the detection of *T. gondii* antibodies, and no cross-reactivity with antibodies against other common canine or feline pathogens was found.

3.4. Sensitivity of the DFICT

To confirm the sensitivity of the DFICT, various concentrations of *T. gondii* antibodies from the diluted dog serum were prepared by dilution in PBS. As shown in Fig. 4, the red line on the testing region can be observed clearly when the dilution of the positive serum samples was up to 1:320, whereas only one test line was observed in the negative control. The same result was obtained with serially diluted positive cat serum against *T. gondii* (data not shown). The result indicated that the strip could detect antibodies in serum samples with a low titer. Similar results were observed upon repeating the tests more than three times, indicating the high reproducibility of the DFICT.

3.5. Stability of the DFICT strip

T. gondii-positive and *T. gondii*-negative sera were retested with

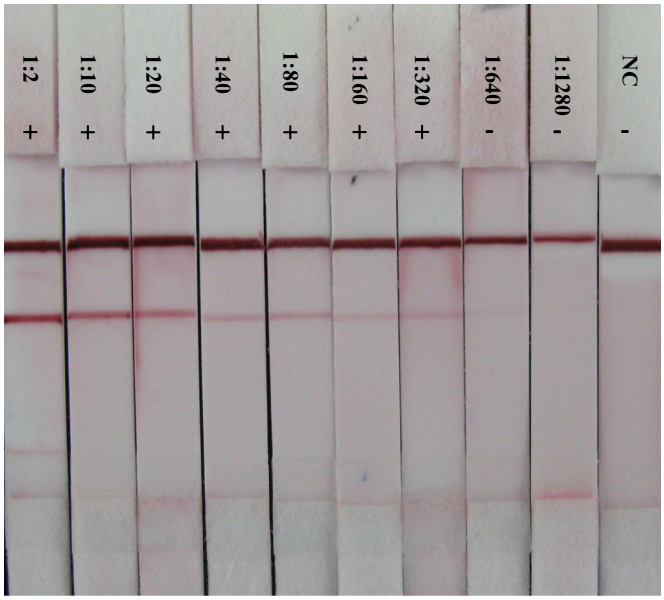

Fig. 4. Sensitivity of the novel DFICT assay. The *T. gondii*-positive dog serum sample was serially diluted in 0.01 M PBS (pH 7.2) from 1:2 to 1:1280 and tested with DFICT strips to determine the assay's sensitivity. *T. gondii*-negative serum was used as the negative control (NC). Three independent experiments were performed in triplicate. Similar results were observed with serially diluted cat serum samples (data not shown).

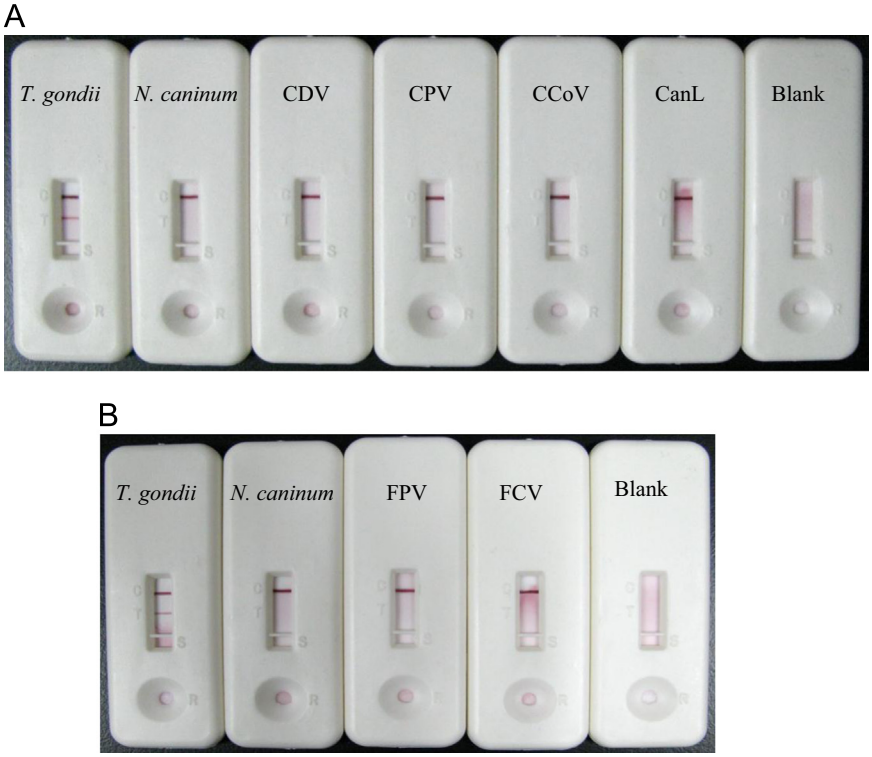

Fig. 3. Specificity of the novel DFICT. A. Positive dog sera against *T. gondii*, *N. caninum*, CDV, CPV, CCoV, and CanL were tested using the DFICT. B. Positive cat sera against *T. gondii*, *N. caninum*, FPV, and FCV were tested using the DFICT. PBS solution was used as the blank control. Similar result patterns were reproduced in repeated experiments.

Table 1
Performance of rapid DFICT strip and the reference ELISA with positive and negative anti-*T. gondii* serum samples.

Serum group	No. of serum samples	No. of samples positive by method	
		Reference ELISA	Rapid DFICT strip
Positive serum samples			
Dog positive controls	25	23	24
Cat positive controls	15	15	13
Total for group	40	38	37
Negative serum samples			
Healthy dog controls	20	1	1
Healthy cat controls	10	0	1
Positive for other pathogens	18	0	0
Total for group	48	1	2

Table 2
Comparison of DFICT with an ELISA kit for dog and cat serum samples.

Group	ELISA results	Rapid DFICT strip results			Kappa statistic[a]
		Positive	Negative	Total	
Dog serum samples	Positive	51	2	53	0.906
	Negative	6	182	188	
	Total	57	184	241	
Cat serum samples	Positive	23	2	25	0.819
	Negative	5	67	72	
	Total	28	69	97	

[a] A kappa statistic of $\geq$ 0.75 represents excellent agreement, 0.40 to 0.75 represents good to fair agreement, and $<$ 0.40 represents poor agreement (Sako et al., 2009).

the DFICT. The gold-conjugated reagents stored at 4 °C for 12 months showed continued sensitivity and could detect up to 1:320 diluted positive dog or cat sera, which is the same sensitivity level of a freshly produced strip. The gold-conjugated reagents left at RT for 6 months were not altered in terms of *T. gondii* detection activity, whereas continual storage at RT for 9 months led to a reduction in sensitivity. Extension of the storage time to 12 months further decreased the sensitivity of the test, only allowing for the detection of 1:160 diluted positive sera of *T. gondii*. Importantly, the specificity of the DFICT for *T. gondii* detection did not change, as evident from the finding that no negative sample became a false positive, regardless of the storage conditions. The results showed that the period of validity of the gold-conjugated reagents, with no loss in sensitivity and specificity for detection of *T. gondii,* was at least 12 months at 4 °C or 6 months at RT.

3.6. Comparison of the rapid test with a reference ELISA.

ELISA is currently considered the commercial standard for detecting antibodies to *T. gondii* (Györke et al., 2011; Wang et al., 2011; Roqueplo et al., 2011; Zhu et al., 2012). The reference ELISA test generated an over-all sensitivity and specificity of 95% (38 of 40) and 97.9% (47 of 48), respectively (Table 1). These results enabled a comparison between the new testing platform with the conventional ELISA approach. The DFICT generated an over-all sensitivity and specificity of 92.5% (37 of 40) and 95.8% (46 of 48), respectively (Table 1), conforming the high sensitivity and specificity of this new test.

To further verify the accuracy of the DFICT for field samples, 241 dog and 97 cat sera were tested, and the results were compared to those obtained with the reference ELISA. As shown in Table 2, among the 241 dog serum samples, 51 of the 57 positive samples determined by the DFICT were also positive via ELISA, while 182 of the 184 negative samples were confirmed as negative by ELISA. Compared with the standard ELISA, the sensitivity and specificity of the DFICT strip for the detection of dog serum samples were 96.2% and 96.8%, respectively. Among the 97 cat serum samples, 23 of the 28 positive cat serum samples determined by the DFICT strips were also determined to be positive via ELISA, while 67 of the 69 negative sera determined by the DFICT strips were confirmed as negative via ELISA. The sensitivity and specificity of the DFICT strip for detection of cat serum samples were 92% and 93.1%, respectively, compared with the standard ELISA. Kappa analysis (Sako et al., 2009) revealed a high degree of agreement between the DFICT and ELISA (κ=0.906 for dog sera and κ=0.819 for cat sera). Due to its good performance, this newly developed method could be used for clinical and field applications for the serological detection of *T. gondii* infection both in dogs and cats.

Although ELISA offers similar detection sensitivity and can be incorporated into an automated procedure, it involves a laboratory, skilled technicians, and special instruments. Measurements take approximately 3.5 h to complete, making the rapid and on-site detection of anti- *T. gondii* antibodies difficult. Compared to ELISA, the DFICT has the following advantages: (1) expertise, experience, and special equipment are not required; (2) it is faster, needing only a 5 min incubation to detect specific antibodies; and (3) it is more economical than ELISA.

4. Conclusion

A novel dynamic flow immunochromatographic test (DFICT) was successfully developed for the serological detection of *T. gondii* infection in both dogs and cats. The detection sensitivity and specificity of the strip test were comparable to that of ELISA -based techniques. The test strip has the advantages of speed, simplicity (i.e., no need to prepare the conjugated and sample pads), ease of use (i.e., not requirement for highly skilled laboratory technicians or sophisticated equipment), and low requirements for serum sample volume (5 μL) without the need for sample processing. The newly developed DFICT represents an innovative and improved option for rapid diagnostic tests.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.04.035.

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