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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.