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BRIEF COMMUNICATION

Evaluation of an immunochromatographic serologic test to detect the presence of anti-*Toxoplasma gondii* antibodies in cats

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Abstract

Background: Toxoplasmosis is a protozoan disease caused by *Toxoplasma gondii*. Different *T. gondii* confirmatory techniques, including serologic methods, are available to detect the presence of the parasite. Among serology techniques, immunochromatographic rapid testing could be a reliable alternative to serologic laboratory techniques.

Objective: This study evaluated a commercial immunochromatographic test (*FAST*est TOXOPLASMA g) in seronegative and seropositive cats.

Methods: Two indirect immunofluorescence antibody reference tests, an in-house technique, and a commercial test were used to classify 292 feline serum samples. The rapid test was evaluated in different groups of cats, including healthy seronegative cats (n = 121), seropositive cats with variable anti-*Toxoplasma* antibodies (n = 146), and cats with positive serologic results for other pathogens (n = 25). The sensitivity, specificity, accuracy, receiver operating characteristic curves, and kappa statistics were analyzed as performance measures.

Results: Of the 292 samples, 146 were classified as *T. gondii* seropositive and 146 as *T. gondii* seronegative. Concordant results were obtained for all samples using immunofluorescence antibody tests. The diagnostic measures of this rapid test showed 98.63% sensitivity and 100% specificity, and 99.32% accuracy. The kappa statistics value was 0.986, and the area under the receiver operating characteristic curve was 0.993.

Conclusions: This rapid test showed diagnostic measurements similar to those of traditional quantitative serologic methods. In situations where laboratory techniques are not available, this test, under clinical conditions, could be a useful alternative to obtain accurate results rapidly.

KEYWORDS
IFAT, rapid test, sensitivity, specificity

Toxoplasma gondii is a protozoan parasite that can infect all warm-blooded animals, such as birds and mammals, including domestic cats and other felids, which are the natural hosts of this coccidian parasite.[1] Cats rarely show clinical signs associated with *T. gondii*, which occur due to the intracellular growth of tachyzoites in different tissues. *T. gondii* infection is more likely to occur in cats with suppressed immune systems, including those under immunosuppressive drug treatment and[2] young kittens and cats affected with *feline* leukemia virus (FeLV) or feline immunodeficiency virus (FIV).[3] Different *T. gondii* confirmatory techniques are available for detecting the presence of the parasite, including the detection of tachyzoites in muscular biopsies, samples obtained by lavage procedures, and body fluids assessed with cytology or molecular tests. Oocysts can also be detected in feces, and serology can be performed to detect antibodies against *T. gondii*,[1] which includes the detection of immunoglobulin G (IgG) and IgM. These methods are only used as an aid for diagnosis, and serology should not be considered a definitive diagnostic tool.[4] Laboratory serologic techniques include immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), modified agglutination test (MAT), and others.[5] IFAT results from tests performed in cats and dogs could be considered representative of the animal's true serologic status.[6] While recent evidence suggests that rapid immunochromatographic tests (ICTs) could be used as an alternative to laboratory techniques and provide rapid results,[7] further evaluation is needed to determine the diagnostic performance of this assay in a clinical setting.[5] In general, ICTs employ a combination of monoclonal antibodies conjugated with colloidal gold particles and recombinant *T. gondii* antigens bound to the solid phase of a nitrocellulose membrane to detect anti-*Toxoplasma* antibodies in feline whole blood, plasma, and serum. Antibodies against *Toxoplasma* present in the sample react in the conjugate pad with mobile monoclonal antibodies to form antibody complexes. These antibody complexes migrate along the nitrocellulose membrane and bind to the fixed *Toxoplasma* antigens, producing a pink-purple-colored test line. Particles that do not bind to the conjugate continue to travel along the membrane and pass through the control line with membrane-fixed control antibodies. The control line indicates that the sample and reagents were properly applied and migrated through the device. The buffer diluent facilitates migration and promotes the binding of antibodies to antigens. Samples with a clear test and control line are classified as positive, while samples with only a control line are classified as negative. It is important to perform this type of serologic analysis because the detection of seropositive animals could be an alternative method to measure the spread of *T. gondii* in the environment[8] in epidemiologic studies.

The present study used well-characterized feline serum samples to assess the diagnostic performance of the commercial *FAST*est TOXOPLASMA g (MEGACOR Diagnostik, Hörbranz, Austria) ICT compared with a commercially available test, namely, the MegaFLUO TOXOPLASMA g (MEGACOR Diagnostik, Hörbranz, Austria) assay, and an in-house IFAT with established performance. Serum samples from the archives (serum bank) of the Clinical Immunology Laboratory, Veterinary Faculty, University of Zaragoza, Spain, were used to evaluate this commercial ICT.

Serum samples from 292 cats were selected for use in the study based on Standards for the Reporting of Diagnostic Accuracy Studies (STARD) guidelines. The previously submitted samples were acquired from January 2019 to August 2021 from the Immunopathology laboratory of the University of Zaragoza, Spain, for diagnostic purposes and seroepidemiologic studies. The average age of the animals was 5 years (range, 1–14 years). Seropositive samples were originally diagnosed by a commercial IFAT (Fluo *Toxoplasma gondii* cat, Agralabo Spa, Scarmargno, Italy). These samples were collected for previous serosurveys (n = 146). The negative control group comprised clinically healthy cats included in the annual screening program (n = 121) and sick cats seropositive for other pathogens to evaluate cross-reactivity (n = 25). The other pathogens included the most prevalent pathogens in the Zaragoza region, such as *Leishmania infantum* (n = 5), *Dirofilaria immitis* (n = 5), *Bartonella henselae* (n = 5), FIV (n = 5), and FeLV (n = 5).[9,10] The inclusion of seropositive samples for other pathogens is commonly performed to evaluate commercial ICTs in small animals.[11]

Serologic status was recorded through a retrospective review of the sample files in the laboratory database. Two aliquots from each serum sample were stored at −20°C until testing. One aliquot was used for diagnostic purposes; the remaining aliquot was stored until testing for research purposes. All the samples used in this study were thawed only *once*.

Because samples were collected for the sole purpose of determining a diagnosis, ethical approval was not required (leftover samples). All examiners were blinded to the previous serologic statuses of these samples.

Rapid ICT (*FAST*est TOXOPLASMA g) was performed according to the manufacturer's instructions. All tests were performed at room temperature. Two laboratory members interpreted the results of all ICTs. Discrepancies between results were resolved by a third observer.

As a first reference method, a commercial IFAT (MegaFLUO TOXOPLASMA g) was performed according to the manufacturer's instructions. All the samples were examined by two different researchers. A positive fluorescence pattern was associated with a dilution of ≥1:50, and no serial two-fold dilutions were performed. Discrepant results were resolved by a third observer. The examiners were blinded to the results of the quantitative serologic tests.

Additionally, an in-house IFAT (98% sensitivity and 96% specificity in the internal protocol of the laboratory) was performed as a reference test using whole formalin-fixed tachyzoites.[12] For the detection of antibodies to *T. gondii*, sera were diluted 1:32 in phosphate-buffered saline (PBS). Briefly, 20 µL of each serum dilution was added to each well. The slides were then incubated for 30 minutes at 37°C in a moist chamber, washed twice with PBS for 5 minutes, and once more with distilled water. After the washing procedure, 20 µL of goat anti-cat IgG-fluorescein isothiocyanate conjugate diluted 1:64 in 0.2% Evans blue was added to each well. The slides were then incubated in a moist chamber at 37°C for 30 minutes in complete darkness and washed again as described above. After the second washing procedure, several drops of mounting medium were placed

TABLE 1 ICT results by serologic status obtained by an in-house IFAT test and commercial IFAT.

Test	Result	Serologic results obtained by in-house IFAT test				Serologic results obtained by MegaFLUO TOXOPLASMA g	
		Negative (n = 146)	Low positive (n = 33)	Medium positive (n = 35)	High positive (n = 78)	Negative (n = 146)	Positive (n = 146)
*FAST*Test TOXOPLASMA g	Test +	0	31	35	78	0	144
	Test −	146	2 (antibody titer1/128)	0	0	146	2

Abbreviations: ICT, immunochromatographic test; IFAT, immunofluorescence antibody test.

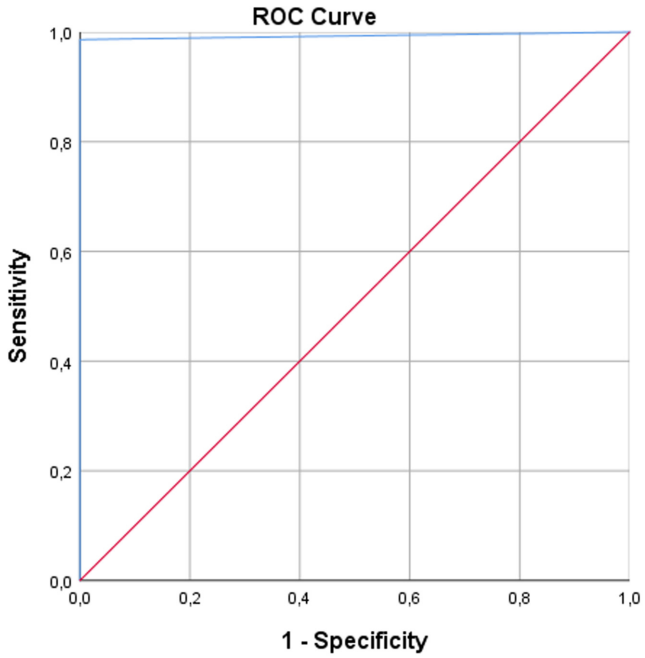

FIGURE 1 The receiver operating characteristic (ROC) curve analysis of the immunochromatographic test (ICT). The area under the curve of the receiver operating characteristic ROC curve analysis combines sensitivity and specificity into one measurement, and the result is a single global measure of diagnostic effectiveness. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The blue line represents the ROC analysis of the *FAST*est TOXOPLASMA g, and the red line represents the reference line.

on the coverslips. The slides were examined under a fluorescence microscope (Leica DM750 RH; Leica Microsystems, Berlin, Germany) at 400× magnification. The fluorescence pattern in each well was compared with that in the positive (tachyzoites showing bright, sharp, and clear yellow-green fluorescence on their membranes) and negative (tachyzoites showing a grayish-dark red color lacking any clear fluorescence) controls included on each slide. Positive control serum was obtained from a cat from Spain diagnosed with *T. gondii* under experimental conditions (presence of tachyzoites in different tissues), while negative control serum was obtained from a healthy, non-infected indoor cat. The cut-off value for positive sera was 1:32. The endpoint titer of positive samples was determined by preparing serial two-fold dilutions of the serum starting from the cut-off value. Sera with antibody titers ≥1:32 and ≤1:128 were classified as low positive, those with an antibody titer ≥1:256 and ≤1:512 as moderately positive, and those with an antibody titer >1:512 as highly positive.

Each quantitative technique was performed independently by different technicians blinded to the results obtained using the other techniques. Additionally, the repeatability of the rapid test was also evaluated, in which four samples with different IFAT titers (<1:32, 1:128, 1:512, and 1:2048) were analyzed six times on the same day by the same operator. IBM SPSS Statistics for Windows, version 22 (IBM Corp., Armonk, NY, USA) was used to construct 2×2 tables and determine the sensitivity, specificity, positive (PPV), and negative (NPV) predictive values; receiver operating characteristic (ROC) curves; and kappa statistics.[13] Binomial confidence limits were calculated for each measure, with 95% confidence intervals (CIs). The obtained data were checked for normal distributions using the Kolmogorov–Smirnov test ($P > 0.05$).

Of the 292 serum samples analyzed, 146 were classified as seronegative, while the remaining 146 were classified as seropositive (≥1:32 cut-off) based on the in-house IFAT test (Table 1). All samples included in this study showed concordant results between the two IFATs. The ICT evaluated in this study showed high sensitivity (98.63%, 95%CI 94.63%–99.76%) and specificity (100%, 95%CI 96.80%–99.94%). The accuracy was 99.32% (95% CI 97.28–99.88) compared with other diagnostic measures. The area under the ROC curve obtained was 0.993 (95%CI 0.982–1.000) (Figure 1). The PPV and NPV were 100% (95%CI 96.76%–99.94%) and 100% (95% CI 94.70%–99.77%), respectively. Finally, the kappa statistic was 0.986 (95% CI 0.663–1.310).

Regarding the repeatability of the ICT, identical results were obtained for all runs and all four samples. Finally, no cross-reactivity was observed in the group of cats seropositive for other pathogens included in this study.

Limited information is available regarding the evaluation of ICTs to detect the presence of antibodies against anti-*Toxoplasma* in cats under clinical practice conditions.[5] A study on the development and evaluation of an ICT based on TgRDT as a recombinant protein to detect antibodies in cats reported a kappa value of 0.88, suggesting very good agreement between the ICT and a commercial ELISA as a reference test.[14] In our study, the kappa value was 0.986, indicating almost perfect agreement ($k > 0.8$) with the in-house IFAT.

Similar results were obtained for the classification of feline serum samples between the in-house IFAT and the ICT in the present study. The main differences between these two serologic techniques are the type of antigen used and the technical methodology used to obtain the results. For IFAT, the antigens are whole formalin-fixed tachyzoites, and a fluorescence microscope is required for slide evaluation by experienced observers. In contrast, ICT uses recombinant proteins as antigens; moreover, the test is simple and easy to perform under clinical conditions and does not require special laboratory equipment, such as a fluorescence microscope.

A potential limitation of this study was the lack of clinicopathologic characteristics (clinical signs and laboratory abnormalities) and unknown toxoplasmosis infection history (clinical suspicion) of the seropositive samples and the retrospective nature of the study (the selection of samples based on previous serologic results). However, the strengths of this study include the determination of the serologic status of the sera, the high number of samples, and the use of other serum samples to evaluate cross-reactivity.

In conclusion, this rapid ICT showed high sensitivity and specificity, with diagnostic measures similar to those of traditional quantitative serologic methods, such as IFAT. In situations where other *Toxoplasma* confirmatory techniques are not available, this test may be an alternative procedure to detect the presence of antibodies against *T. gondii*.

DISCLOSURE

The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

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