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Comparison of Diagnostic Technique for Detection of *Toxoplasma gondii* Infection in Dairy Cows in Thailand

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ABSTRACT

Toxoplasma gondii infection in animals reared for human consumption has an impact on the economics of production and on public health, since this pathogen has been the causative agent of abortion and able to transmit to humans. Efficient diagnosis is needed to identify infected animals in order to reduce the economic damage and lower the risk of infection to humans. The objective of this study was to evaluate a practical diagnostic tool to use for routine diagnosis by veterinary clinicians. Serum samples from 50 dairy cows were evaluated by the indirect fluorescence antibody test (IFAT). Four tests for the diagnosis of toxoplasmosis: enzyme linked immuno sorbent (ELISA), latex agglutination (LAT), polymerase chain reaction (PCR) and loop mediated amplification (LAMP) were compared to an indirect fluorescence antibody test (IFAT). The highest sensitivity rates were achieved with LAT (100%). The specificity varied with values of 82.6% (LAMP), 91.3% (LAT), and 100% (PCR). The results demonstrated that LAT had a higher sensitivity and specificity compared to IFAT as a gold standard for the diagnosis of *T. gondii* infections and indicated that LAT should be used as the routine diagnostic test for the detection of *T. gondii* infection in dairy cows in Thailand.

Key words: *T.gondii*, IFAT, ELISA, LAT, PCR, LAMP

INTRODUCTION

Toxoplasma gondii is an important zoonotic protozoan parasite with a worldwide distribution. It is capable of infecting all warm-blooded animals and estimated to infect from 4 to 77% of the human population (Tenter *et al*., 2000). While *T. gondii* can be transmitted directly by animal-human contact or through contact with contaminated feces, soil and herbage, it can also be transmitted through contaminated food and water. In animals, infection not only results in significantly reducing reproduction and hence causing economic losses, but also has implications for public health, since consumption of contaminated meat or milk can facilitate zoonotic transmission.

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Serological studies in Thailand have found evidences of its widespread occurrence in humans (Sukthana *et al*., 2001), dogs and cats (Nishikawa *et al*., 1989; Sriwaranard *et al*., 1981; Jittapalapong *et al*., 2007), goats (Jittapalapong *et al*., 2005), dairy cows (Jittapalapong *et al*., 2008), tigers (Thiangtum *et al*., 2006), elephants (Tuntasuvan *et al*., 2001) and rodents (Jittapalapong *et al*., 2006).

The diagnosis of toxoplasmosis classically relies on serology and the demonstration of the pathogen in animal samples. Sabin and Feldman (1948) first described the dye test for the detection of toxoplasmic-specific antibodies. It is a highly specific and sensitive test and still used as the referent method for the diagnosis of toxoplasmosis. Therefore, an alternative method, which is highly sensitive, easy to perform and not time-consuming is needed. Latex agglutination, indirect haemagglutination and indirect immunofluorescence have been employed. However, though the latex agglutination test was found to be highly sensitive in previous studies from areas with a high prevalence of *Toxoplasma* infection, it has never been assessed in populations with comparatively low prevalence, such as Thailand. The PCR technique for the detection of *Toxoplasma* has been described and evaluated by different groups (Burg *et al*., 1989).

The current study evaluated the results of IFAT, ELISA, LAT, PCR and LAMP as laboratory tests for the diagnosis of anti-*T. gondii* antibodies in paired sera and whole blood samples for molecular assay.

MATERIALS AND METHODS

Diagnosis of *T. gondii* infection

LAMP (loop-mediated isothermal amplification)

The LAMP reaction was conducted according to previous reports described by Zhang *et al*. (2009). LAMP was carried out in a total 25-μL reaction mixture. The primer mix (0.9 μL) contained 40 pmol of FIP and BIP each, 5 pmol of F3 and B3 each, 20 pmol of LB and FB each, 12.5 μL of reaction buffer (1.6 mol betaine, 40 mmol Tris–HCl [pH 8.8], 20 mmol KCl, 20 mmol/L $(NH_4)_2SO_4$, 2.8 mmol each deoxyribonucleotide triphosphate (dNTP), 0.2% Tween 20, 16 mmol $MgSO_4$), 1 μL (8 U) of *Bst* DNA polymerase (Eiken, Japan), 1 μL of DNA and 8.7 μL of distilled water. The mixture was incubated at 63° C for 1 h and then electrophoresis was performed with 2% agarose gel.

PCR (Polymerase chain reaction)

The PCR was performed essentially as described by Homan *et al*., 2000. Briefly, the 529 bp gene was amplified from each sample by conventional PCR, using primers TOX4 (CGCTGCAGGGAGGAAGACGAAAGTTG) and TOX5 (CGCTGCAGACACAGTGCATCT-GGATT). Each PCR was consisted of 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, with an initial denaturation step consisting of incubation at 94°C for 5 min and a final extension step consisting of incubation at 72°C for 10 min also included. After PCR amplification, the PCR products were run with 1% agarose gel electrophoresis and stained with ethidium bromide (10 μg/ml) for visualization.

Latex agglutination test

The presence of *T. gondii* antibodies was analyzed by a latex agglutination test (LAT) kit (Toxocheck-MT; Eiken Chemical Company, Tanabe, Tokyo, Japan) following the protocol of Tsubota *et al*., (1977 a and b). The procedure described in a previous report (Jittapalapong *et al*., 2005; 2007) was accurately followed. According to the kit instructions, the cut-off titer for this test was at 1:64. The obtained results were analyzed by the Chi-square test, and the level of significance was set at $p < 0.05$.

IFAT

Antigen slides were incubated with cow sera diluted 1:100 in 5% bovine serum albumin (BSA) with PBS for 1 h at 37°C and then with a fluorescein isothiocyanate-labeled goat anti-bovine IgG diluted 1:200 in 5% BSA with PBS. Positive and negative control sera were included on each slide. Slides were examined under fluorescence microscopy and only a bright, linear peripheral fluorescence of the tachyzoites was considered positive.

ELISA

The ELISA was performed in flat-bottomed 96-well microplates (Nunc, Denmark). The purified GST-TgSAG1t was diluted to an optimal concentration (2 μg/ml) in a 50 mM carbonate-bicarbonate buffer (pH 9.6), of which 50 μl was added separately to duplicate wells for each sample. Coated plates were incubated at 4°C overnight. After the unabsorbed antigen was discarded, the wells were blocked with PBS containing 3% skim milk (blocking solution, 100 μl / well) at 37°C for 1 h. Then the plates were washed once with PBS containing 0.05% Tween 20 (PBS-T). Fifty microliters of serum diluted in the blocking solution (1:100) was added to each well and incubated at 37°C for 1 h. After incubation, the wells were washed six times with PBS-T and subsequently incubated with 50 μl of goat anti-bovine IgG- horseradish peroxidase conjugate (ICN Biochemical, USA) (1:4,000) at 37°C for 1 h. After six washes, 100 μl of substrate solution (0.05% 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid), 0.2 M sodium phosphate, 0.1 M citric acid, 0.003% H_2O_2) was added to each well. After 1 h of reaction at room temperature, the optimal density (OD) was read at 415 nm using an MTP-120 ELISA reader (Corona Electric, Japan). The ELISA titer was expressed as the reciprocal of the maximum dilution that showed an ELISA value equal to or greater than 0.1, which is the difference in absorbance between that for the antigen (GST-TgSAG1t) well and that for the control antigen (GST) well. The cut-off point of 0.1 was the mean OD for negative sera plus three standard deviations.

RESULTS

Serological examination for the detection of *T. gondii* antibodies

Examination of 50 serum samples from dairy cows through IFAT, LAT and ELISA revealed that 27 (54%), 27 (54%) and 18 (36%), respectively, had antibodies against *T. gondii* (Table 1).

Comparative studies of the different diagnostic tests for detection of *T. gondii*' DNA

Sensitivity and specificity calculations of the molecular technique revealed that LAT had the highest sensitivity (100%), followed by ELISA (55.5%), LAMP (14.8%) and PCR, which demonstrated the lowest sensitivity (0%). For specificity, PCR had the highest specificity (100%)

Table 1 Sensitivity, specificity and prevalence of *T. gondii* infections using different diagnostic tests compared with IFAT.

Test	$+^{ve}$	$-^{ve}$	Sensitivity (%)	Specificity (%)	+Predictive value	Prevalence (%)
IFAT	27	23	100	100	100	54
LAT	29	21	100	91.3	92.9	54
ELISA	18	32	55.5	65.2	65.2	46
LAMP	8	42	14.8	82.6	50	16
PCR	0	50	0	100	0	0

compared to LAT (91.3%), LAMP (82.6%) and ELISA (65.2%).

DISCUSSION

The variation between the results obtained from the same cow sera using different diagnostic tests might have been due to differences in the sensitivity and specificity of the tests used. IFAT, approved by OIE (OIE, 2004), the highly specific test, is a complicated test, requiring fluorescently labeled conjugated and special equipment (Shaapan *et al.*, 2008). LAT demonstrated great sensitivity (100%) and is quantitative, low cost and may be adopted easily, but requires further confirmation testing due to its lower specificity.

Dubey and Beattie (1988) suggested that a dye test was the most specific test for *T. gondii* and that it was considered the gold standard by which all other tests should be judged. However, OIE recommended IFAT as the standard test for diagnosis in animals, since the dye test had the major disadvantage of requiring the use of live organisms and human serum from healthy individuals as an accessory factor. Therefore, the dye test has been replaced with other tests, such as IFAT.

CONCLUSION

The results of the present work demonstrated the benefits of using the more sensitive and somewhat specific LAT for the detection of *T. gondii* antibodies in cow sera. In addition, the serological tests used depended on antigen prepared from locally isolated *T. gondii*, which were much cheaper when compared with the expensive commercial kits.

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