Validation of the modified agglutination test for the detection of *Toxoplasma gondii* in free-range chickens by using cat and mouse bioassay

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SUMMARY

The modified agglutination test (MAT) is one of the most commonly used tests for the detection of antibodies to *Toxoplasma gondii* in animal and human sera. The objective of the present study was to evaluate the diagnostic accuracy of the MAT and bioassay in free-range/backyard (FR) chickens (*Gallus domesticus*). Previously-published *T. gondii* test results from 2066 chickens from 19 countries were compiled for the present study. The frequency of isolation of *T. gondii* increased for MAT titres between 1:5 and 1:160, and ranged from 61 to 75% for antibody titres of 1:160, 1:320, and ⩾1:640. Twenty-three cats fed pooled hearts from a total of 802 FR seronegative (MAT, <1:5) chickens from several countries did not excrete oocysts, indicating a high negative predictive value of MAT because FR chickens would have been exposed to many microbes; cats are the most sensitive indicators of *T. gondii* infection in tissues and can excrete millions of oocysts after ingesting even a few bradyzoites. Of the 29 cats in this study, six cats, fed hearts pooled from 15–122 FR chickens, excreted oocysts; but these identifications were likely related to misidentification or prozone. Results of the present study support the validity of MAT for the detection of *T. gondii* infection in chickens.

Key words: *Toxoplasma gondii*, chickens, modified agglutination test, bioassay.

INTRODUCTION

Toxoplasma gondii infects virtually all warm-blooded animals, including humans; and infections are worldwide (Dubey, 2010*a*). Serological tests are often used to determine exposure to the parasite. Unlike confirmations of *T. gondii* infections in humans, the serological status of animals can be verified by attempting to isolate, with bioassay techniques, viable parasites from animals at death.

Among many serological tests available for the detection of *T. gondii* antibodies in human or animal sera, the modified agglutination test (MAT) is simple, easy to perform, does not require special equipment or species-specific reagents, and can be used for all species including humans (Dubey, 2010*a*). Antigen for the MAT is stable for months, and reagents are commercially available.

A highly sensitive method of detecting *T. gondii* bradyzoites in tissues is bioassay in cats. Experimentally, cats fed as few as one bradyzoite may excrete large numbers of oocysts in their feces (Dubey, 2001), as *T. gondii* multiplies extensively in the intestine of the cat. This many-fold amplification greatly facilities the detection of *T. gondii* in test samples with small numbers of tissue cysts, and cats have been used to detect viable *T. gondii* in meat because larger volumes of tissues (250 g or more) can be fed to cats than can be assayed in mice (Dubey *et al.* 1995; Dubey *et al.* 2005*a*, *b*, *c*, *d*, *e*, *f*, *g*, *h*).

Information on accuracy of diagnostic tests for diagnosis of toxoplasmosis in food animals is limited. In a previous study, 1000 hearts of naturally-exposed adult sows were bioassayed in 10 000 mice (10 mice for each heart, irrespective of serological status) and 165 cats (only hearts with low antibody titres) for viable *T. gondii*; and sera removed from the hearts were tested for *T. gondii* antibodies using the MAT (Dubey *et al.* 1995). In that study, viable *T. gondii* was isolated from 170 pigs (108 by bioassay in mice and 62 by bioassay in cats). The isolation of *T. gondii* generally increased with antibody titre. However, 29 of 170 isolates were from pigs with MAT titres <1:20. Seventeen of these 29 pigs were found to have MAT titre of 1:10; a 1:5 dilution was not tested (Dubey *et al.* 1995). Another drawback of that study was that pigs were from one geographical area (Iowa) and of one age group (adults), and only from sows.

In 2002, an international survey of *T. gondii* infection in free-range (FR) chickens (*Gallus domesticus*) was initiated with the ultimate objective of studying the genetic diversity of *T. gondii* on a worldwide basis (Dubey *et al.* 2002; Lehmann *et al.* 2006;

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Dubey, 2010*b*; Dubey *et al*. 2012; Shwab *et al*. 2014). FR chickens (>2000) from 19 countries were bioassayed for the isolation of viable *T. gondii* (Table 1). Because these studies are very expensive to conduct with respect to money, time and resources, we have summarized the data in the present paper, with the objective of evaluating MAT accuracy.

MATERIALS AND METHODS

Serological and parasitological examination of naturally-exposed chickens

Previously-published *T. gondii* test results from 2066 chickens from 19 countries were compiled for the present study (Table 1). Most of the chickens were raised in the backyards of homes, except those from Austria that were raised in a commercial FR system (Table 1). The selection of properties was based on the owner's willingness to participate.

In these studies, male and female chickens, mostly adults (>6 months), were slaughtered humanely; and their sera and tissues (mainly hearts) were transported on ice to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, Maryland, where all testing was performed. Up to 1 week elapsed between killing of chickens and receipt of samples at APDL. During that time, samples were kept cold but not frozen.

Sera of chickens were first screened for *T. gondii* antibodies, usually using four screening dilutions (1:5, 1:10, 1:20 and 1:40) for the MAT (Dubey and Desmonts, 1987), depending upon workload in the laboratory and the number of chicken samples received for testing on a given day (Table 1). Any positive samples at the screening dilutions were further tested in serial 2-fold dilutions to a maximum dilution of 1:1280.

For isolation of *T. gondii*, tissues were bioassayed in mice or cats or both. Because the number of *T. gondii* in tissues of latently-infected animals is low, a digestion method was used to concentrate *T. gondii* in the inocula, following the procedure described in detail by Dubey (2010*b*). Hearts from seropositive chickens were bioassayed individually in mice (Table 1). In brief, the myocardium (approximately 10 g) was homogenized in 50 mL saline (0·85% aqueous NaCl), to which double strength pepsin-HCl mixture was added (Dubey, 2010*a*). The homogenized solution was then incubated at 37 °C in a shaking water bath for 1 h then filtered and centrifuged at 400 ***g*** at 10 °C, after which the homogenate was suspended in antibiotic saline (Dubey, 2010*a*). The homogenate was inoculated into 4–5 Swiss Webster mice. The inoculated mice were observed for 6 weeks or more, and each mouse was tested for *T. gondii* infection serologically and parasitologically. The mice were considered infected with *T. gondii* only when viable tachyzoites or tissue cysts were demonstrated (Dubey, 2010*a*). Details are provided in publications indicated in Table 1.

The initial plan was to bioassay tissues of seronegative chickens (MAT titres <1:5) in cats and bioassay hearts of seropositive chickens (titres ⩾1:20) individually in mice. Because *T. gondii*-free cats are expensive, pooled hearts from a total of 1018 FR seronegative chickens (with 12–137 chickens per pool) were used for bioassay in cats (Table 2). Cats were fed chopped up chicken hearts over a period of 1–3 days. Feces of cats were subsequently examined for the presence of *T. gondii* oocysts 3–14 days after feeding these hearts, following protocols described by Dubey (2010*a*). All cat feces collected for each 24-h period were mixed with sucrose solution and centrifuged at 400 ***g***, and a drop from the float was examined microscopically for *T. gondii* oocysts. Float from each cat feces was mixed with water and centrifuged, and the sediment was incubated in 2% sulphuric acid for sporulation of oocysts and subsequently bioassayed in mice, as described in detail by Dubey (2010*a*). Final results were based on bioassay in mice to ensure that few oocysts were not missed by microscopical examination.

RESULTS

The frequency of isolation of *T. gondii* from chicken heart tissue using mouse bioassay generally increased with rising MAT antibody titres in chickens. The isolation rate with respect to antibody titres were: 0·6% for titre <5(6 of 1025 chickens), 15·2% for titre 5 (16 of 105 chickens), 11·4% for titre 10 (9 of 79 chickens), 42·9% for titre of 20 (42 of 98 chickens), and 59·9% for titres of 40 or higher (455 of 759 chickens).

For the cat bioassay, 26 of 29 cats fed hearts pooled from a total of 1021 chickens with MAT < 1:5 excreted oocysts. Of these 29 cats, 23 cats that did not excrete oocysts had been fed hearts pooled from 802 chickens.

DISCUSSION

Under natural conditions, FR chickens are exposed to many microbes, some closely related to *T. gondii* (e.g. *Sarcocystis, Eimeria, Cryptosporidium*); the present study provided an opportunity to evaluate the diagnostic accuracy of MAT under these conditions. The accuracy estimates apply only to the source populations, but estimates may be different in intensively-reared chickens with lower exposure frequencies to closely-related parasites. However, in experimentally-infected chickens, there was no reactivity previously reported between the *T. gondii* antigen and serum antibodies from chickens infected with *Eimeria acervulina* and *Eimeria tenella* (Dubey *et al*. 1993). As noted earlier, bioassay in cats is a

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Table 1. Summary of isolation of viable *Toxoplasma gondii* from tissues of 2066 naturally infected free range chickens from 19 countries

			MAT antibody titre of chicken									
Country (Reference)	No	% prevalence	<5	5	10	20	40	80	160	320	640	1280
Brazil (da Silva *et al*. 2003)	49	24·4	**0/20**[a]	0	0	0	1/1	3/6	18/22	ND	ND	ND
Brazil (Dubey *et al*. 2002, 2003*a*)	96	69·7	0	5/19	0/3	5/6	2/6	15/15	9/10	31/37	ND	ND
Egypt (Dubey *et al*. 2003*b*)	98	20·4	**0/34, 1/15**	1/11	0/4	2/4	1/8	5/10	3/4	7/8	ND	ND
USA (Dubey *et al*. 2003*c*)	83	13·2	**0/63**	3/5	1/5	1/1	1/1	2/4	3/4	ND	ND	ND
Argentina (Dubey *et al*. 2003*e*)	19	57·8	ND	0/1	1/1	4/6	2/2	2/2	0/1	1/3	0	1/3
Brazil Dubey *et al*. 2003*d*)	40	35·0	**0/12, 1/12**	1/3	1/1	2/3	1/1	2/2	2/2	1/1	1/1	2/2
Mexico (Dubey *et al*. 2004*b*)	55	14·2	**0/42**	0	0/3	3/4	1/1	0/1	2/2	0/2	0	0
Israel (Dubey *et al*. 2004*c*)	96	21·8	**1/26, 1/25**	1/3	0/2	0	1/5	2/5	2/3	13/27	ND	ND
Peru (Dubey *et al*. 2004*a*)	50	20·0	**0/37**	0/1	0	1/3	0	0	2/2	7/7	0	0
Congo (Dubey *et al*. 2005*e*)	50	20·0	**0/25**	0/7	1/7	4/6	1/1	0	4/4	0	ND	ND
Grenada (Dubey *et al*. 2005*a*)	102	35·2	**0/49**	0/6	1/4	2/4	4/4	11/15	9/9	3/5	4/4	2/2
Argentina (Dubey *et al*. 2005*g*)	60	28·3	**0/39**	0	1/1	1/2	1/1	1/1	4/4	2/5	3/3	4/4
Sri Lanka (Dubey *et al*. 2005*h*)	100	11·0	**0/61**	1/8	1/8	2/4	2/5	0/5	3/5	1/2	1/2	0
Austria (Dubey *et al*. 2005*b*)	505	11·0	**0/37, 0/137, 1/122**	ND	0/3	2/33	11/46	6/39	16/29	16/43	4/16	ND
Colombia (Dubey *et al*. 2005*c*)	64	37·5	**0/16, 1/16**	0/4	0/3	1/1	1/1	7/8	8/8	2/3	4/4	ND
Venezuela (Dubey *et al*. 2005*f*)	47	27·6	**0/30**	0/1	0/2	0	3/3	2/2	2/2	2/3	2/2	2/2
Brazil (Dubey *et al*. 2006*a*)	50	48·0	**0/17**	2/3	0/2	1/1	0/1	2/2	10/14	5/5	2/2	2/3
Portugal (Dubey *et al*. 2006*e*)	99	16·1	**0/38**	0/8	0/6	2/3	1/23	4/5	3/4	3/8	3/4	ND
Costa Rica (Dubey *et al*. 2006*c*)	143	21·6	**0/48, 0/28**	0/15	1/5	1/2	1/3	4/8	8/16	5/5	9/10	3/3
Chile (Dubey *et al*. 2006*b*)	70	31·4	0/7, **0/16**	0/6	0/4	1/4	1/3	5/9	3/4	6/9	5/7	1/1
Nicaragua (Dubey *et al*. 2006*d*)	98	47·9	**0/24**	0	1/8	5/7	4/9	6/11	22/28	4/6	4/4	1/1
USA (Dubey *et al*. 2007)	11	100	0	0	0	0	1/1	0	0	3/3	7/7	0
Italy (Dubey *et al*. 2008)	19	10·5	**0/14**	0/1	0	0/1	0	1/2	0	1/1	ND	ND
Poland (Dubey *et al*. 2008)	20	10·0	**0/15**	1/1	0/3	0	0	1/1	0	0	0	0
Brazil (Dubey *et al*. 2010)	42	50·0	0	1/2	0/4	2/3	2/6	5/6	3/5	2/3	8/13	0
Total	2066		6/1025	16/105	9/79	42/98	43/132	86/159	136/182	115/186	57/79	18/21

ND, no data

[a] Figures in bold – bioassay in cats.

Table 2. Antibody titres and isolation of viable *Toxoplasma gondii* by bioassay in mice from naturally exposed free-range chickens from 19 countries

MAT titre	No. of chickens bioassayed	No. of positive chickens	% seropositive (95% confidence interval)
<5	1025	6	0·6 (0·2–1·3)
5	105	16	15·2 (10·7–26·2)
10	79	9	11·4 (5·3–20·5)
20	98	42	42·9 (30·5–49·7)
40	132	43	32·6 (22·3–38·7)
80	159	86	54·1 (45·7–61·7)
160	182	136	74·7 (66·6–79·9)
320	186	115	61·8 (53·9–68·3)
⩾640	100	75	75·0 (65·3–83·1)

MAT, modified agglutination test.

Table 3. Isolation of viable *Toxoplasma gondii* by bioassay in cats from naturally exposed free-range seronegative (modified agglutination test titre, <1:5) chickens (heart tissue)

Pool sizes of chicken hearts (total number of chickens bioassayed)	Cats fed chickens	Cats excreting oocysts
20, 34, 63, 12, 42, 37, 25, 49, 39, 61, 37, 137, 16, 30, 17, 38, 48, 20, 8, 16, 24, 14, 15 (total = 802)	23	0
15, 122, 12, 16, 25, 26 (total = 216)	6	6

highly sensitive indicator of *T. gondii* infection; and the observation, that 23 cats did not excrete oocysts when fed pooled hearts from a total of 802 seronegative chickens (MAT < 1:5), is important evidence supporting high negative predictive value of the MAT. The excretion of oocysts by 6 cats that were fed hearts from a total of 216 seronegative chickens (MAT < 1:5; pools of 12–122 chickens) may have occurred because there was a mismatching of serum and tissue, because the chickens had not yet seroconverted, because the antibodies had declined to undetectable levels, or due to prozone (Table 3).

The criterion for accuracy analysis was viable *T. gondii* infection. Our data might have been different if all samples were bioassayed in cats rather than mice, and if more than one tissue type was used for bioassay. Although it is likely that *T. gondii* was present in other organs from the seropositive chickens, in the present study, the choice of only heart samples for bioassay may have led to an underestimation of MAT accuracy. Dubey (2010*b*) found that, among 2066 chicken hearts and brains of 136 naturally–infected chickens from various countries bioassayed in mice, viable *T. gondii* was isolated from heart tissue (89·5%) and brain tissue (49·2%), indicating false negative results in 10·5% of those bioassays from heart tissue. Isolation rates among different muscle sources also varied; *T. gondii* was previously isolated from 44·1% of 34 leg muscles *vs* 18·6% of pectoral muscle (Dubey, 2010*a*). For the present study, it would have been ideal to bioassay tissues of all chickens both in cats and mice, but it would have been extremely expensive. Recently, tissues of 26 MAT-seropositive naturally-exposed chickens from 3 New England farms in the USA were bioassayed both in cats and mice; and viable *T. gondii* was isolated from hearts (100%), brains of 2 (7·7%), and pectoral muscles of 11 (42·3%) (Dubey *et al*. 2015). Furthermore, the amount of tissue bioassayed in mice was not a factor in this study because all of the brain tissue, all of the myocardium and 25 g of leg muscle from the 26 chickens were digested in pepsin and digests were inoculated into 5 mice for each tissue type. For bioassay in cats, muscle (250–500 g) from the remainder of the chicken carcasses was fed to 1 cat for each of the 26 chickens; and 23 (88·5%) of 26 cats excreted oocysts. Results of this study indicate that both the tissue and the amount of tissue (25 g for bioassay in mice *vs* 250 g for cat bioassay) and the type of bioassay (cats *vs* mice) bioassayed may be important determinants, but heart is the tissue of choice for isolating *T. gondii* from chickens.

Data in the present study were obtained from 2066 chickens from 19 countries and were based on the isolation of viable *T. gondii* in mice and not upon the seroconversion in bioassayed mice as used by Casartelli-Alves *et al*. (2014, and personal communication to JPD July 12, 2014). They had estimated accuracy of serological tests, histopathology and immunohistochemistry using mouse bioassay results of 135 adult chickens in Brazil as the reference standard. Antibodies to *T. gondii* were found in 82 chickens by indirect fluorescent antibody test (⩾1:16), in 81 chickens by ELISA, in 67 chickens by MAT (⩾1:16), and in 49 chickens by indirect

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hemagglutination test (IHAT, ⩾1:16). In their bioassay tests, mice inoculated with tissues of 54 of the 135 chickens had detectable antibodies to *T. gondii* using IHAT (1:16), and mice inoculated with tissues of 39 of the 135 chickens had viable *T. gondii*. Based on results that considered the 54 chickens as bioassay positive, the sensitivity and specificity of MAT were calculated as 76 and 68%, respectively. However, seropositivity only indicates exposure and does not equate with infectivity, especially if a low titre (1:16) in the IHAT is considered positive (Dubey, 2010*a*).

With respect to food safety, public health and epidemiology, the prevalence of *T. gondii* in commercially-raised chickens has not been extensively evaluated. In the USA, more than four billion chickens are slaughtered for food annually, and most of them are broilers less than 8 weeks of age. In one study, *T. gondii* was not isolated by bioassay in cats fed breast muscle samples from 2094 chickens from retail meat stores in the USA; however, antibodies to *T. gondii* were detected in an ELISA performed on their meat juice (Dubey *et al*. 2005*d*). Freezing of meat might have affected the infectivity of *T. gondii* in the former study. In many countries, FR chickens are slaughtered for food at home under unsupervised conditions, and contamination of humans during slaughter and cooking of chickens could be a source of *T. gondii* infections. In addition, scavenging of improperly disposed viscera of infected chickens is an important source of infection for cats and of further environmental contamination via oocysts shed by cats (Ruiz and Frenkel, 1980). Moreover, prevalence of *T. gondii* in FR chickens may be indicative of soil contamination because chickens feed from the ground.

Results of the present study support the validity of MAT for the detection of *T. gondii* infection in chickens. For epidemiological studies, even low MAT titres (1:5) should be considered as indicative of possible *T. gondii* exposure of chickens.

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