International Journal for Parasitology: Parasites and Wildlife 18 (2022) 287–291

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Comparison of a commercial ELISA and indirect hemagglutination assay with the modified agglutination test for detection of *Toxoplasma gondii* antibodies in giant panda (*Ailuropoda melanoleuca*)

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ARTICLE INFO

Keywords:
Toxoplasma gondii
Giant panda
Modified agglutination test
Indirect hemagglutination assay
ELISA

ABSTRACT

Toxoplasma gondii is a worldwide-distributed zoonotic protozoan parasite which causes toxoplasmosis and has a significant effect on public health. In the giant panda (*Ailuropoda melanoleuca*), toxoplasmosis can cause asymptomatic infections, reproductive disorder and even death, which poses a serious threat to the conservation of this rare protected species. Therefore, serological investigation of *T. gondii* is essential to understanding its risk to giant pandas, however, there are no specific testing kits for giant pandas. Previous research has used MAT as the reference method for screening *T. gondii*, to investigate this further, this study focused on the agreement comparing of MAT with ELISA and IHA tests for detecting *T. gondii* antibodies in 100 blood samples from 55 captive giant pandas in Chengdu, China. The results showed 87.0%, 87.0%, 84.0%, samples were sero-positive for *T. gondii* using ELISA (kits a, b, c), respectively, while MAT and IHA tests were 84.0% and 9.0% sero-positive, respectively. There was no significant difference between MAT and the three ELISA kits and these two methods had substantial agreement ($0.61 < \kappa \leq 0.80$). Meanwhile, there was a significant difference ($P < 0.001$) between MAT and IHA, and these two methods had only a slight agreement ($\kappa \leq 0.20$). The relative sensitivity of the ELISA (kits a, b, c) were 89.0%, 91.5% and 95.1%, and the specificity were 86.7%, 80.0% and 80.0%, respectively, which showed these three ELISA kits all had great accuracy. It is suggested that MAT is the recommended test method for primary screening *T. gondii* in giant pandas and then verified by ELISA.

1. Introduction

Toxoplasma gondii is a globally distributed obligate intracellular protozoan, known to infect wildlife, domestic animals and humans, (Dubey, 2022). Toxoplasmosis is a disease that results from infection with the *T. gondii*, and continues to be a public health concern. Felids are the only definitive host of *T. gondii* and shed oocysts in their feces which can contaminate the environment (Dubey, 2009), while various mammals, including giant panda (*Ailuropoda melanoleuca*), serve as the intermediate host.

As an iconic "flagship" species for wildlife conservation, the giant panda is considered a national treasure in China and also well-known around the world (Peng et al., 2007). The giant panda is currently categorized as "vulnerable" by the International Union for Conservation of Nature (IUCN, https://www.iucn.org/). However, due to the destruction of their natural habitat, infectious diseases and low reproductive rates, the giant pandas are facing continued threats within both the *in-situ* and *ex-situ* populations. Recently, a captive giant panda died because of acute fatal *T. gondii* infection in a zoo in China, which showed that toxoplasmosis poses a threat to the health of giant pandas (Ma et al.,

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https://doi.org/10.1016/j.ijppaw.2022.07.001
Received 24 May 2022; Received in revised form 5 July 2022; Accepted 6 July 2022
Available online 16 July 2022

2015). Hence, it's necessary to carry out the investigation of *T. gondii* infection in the giant pandas.

The detection of the *T. gondii* infection in animals primarily relies on serological assays. While numerous studies have been performed on the sero-prevalence of *T. gondii* in wildlife, in regards to the giant panda, there is one previous investigation of note. Zhong et al. (2014), screened 69 captive giant pandas and found that one individual tested sero-positive for *T. gondii*.

The accuracy of different serological methods is usually assessed by comparing results with other serological tests, especially for wildlife (Werre et al., 2002). Among the serological tests, modified agglutination test (MAT) and enzyme linked immunosorbent assay (ELISA) have been widely used both in epidemiological surveys for screening of *T. gondii* infection in wildlife (Stensgaard et al., 2022) and domestic animals (Moghazy et al., 2011; Dubey et al., 2005). Consequently, it's necessary to select a suitable serological detection method of *T. gondii* antibodies in giant pandas. Herein, four commercial tests were compared with MAT for the detection of *T. gondii* antibodies for screening out a suitable diagnosis of *T. gondii* infection in giant pandas.

2. Materials and methods

2.1. Sample collection

In this study, a total of 100 blood samples from 55 captive giant pandas were collected at the Chengdu Research Base of Giant Panda Breeding, Sichuan, China, from May 2013 to November 2021. The animal handing procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Chengdu Research Base of Giant Panda Breeding (NO. 2020006). The samples were collected from the forelimb vein of giant panda without anesthesia, taken in the anticoagulant-free vacuum blood collection tube and then centrifuged at 3500 rpm for 10 min for serum separation after coagulation, and the sera were stored at −80 °C after divided and labeled for the following test.

2.2. Detection of antibodies to T. gondii

2.2.1. Modified agglutination test (MAT)

All the 100 sera samples were tested for *T. gondii* antibodies using the commercial *T. gondii* MAT kit (University of Tennessee Research Foundation, Technology Transfer & Licensing, Memphis, USA) with a cut-off titer of 25, the experimental procedure was performed according to the manufacturer's instructions. Briefly, the experiment was carried out on 96-well U-bottom microtiter plate, the samples were diluted in twofold serial dilutions from 1: 25 to 1: 200, then the premixed reaction reagent was added to each well, the plate was then placed in a humid incubator at 37 °C for 24 h. A positive result was noted when the reaction showed a layer of agglutinated tachyzoites covering more than half of the reaction well's bottom. A negative result was noted when the tachyzoites sedimentary formed a small button at the reaction well's bottom.

2.2.2. Indirect hemagglutination test (IHA)

All the samples were tested for immunoglobulin antibodies to *T. gondii* using the commercial IHA kit (Lanzhou Shouyan Biotechnology Co., Ltd, Lanzhou, China.) with a cut-off titer of 4, the experimental procedure was performed according to the manufacturer's instructions. The experiment was carried out on 96-well V-bottom microtiter plate, the samples were diluted in twofold serial dilutions from 1: 4 to 1: 256, then the antigen diagnostic fluid prepared on advance into each reaction well was added, the plate was then placed in an incubator at 37 °C for 3 h. The positive sample showed no deposits and were uniformly distributed at the reaction well's bottom, and negative samples showed a clear agglutination point at the reaction well's bottom.

2.2.3. Enzyme linked immunosorbent assay test (ELISA)

For screening suitable T. gondii ELISA kits for giant panda, we selected ELISA(a) (kit multi-species ID.Screen® Toxoplasmosis Indirect, IDVET, Grabels, France), ELISA(b) (Haitai Biological Pharmaceuticals Co., Ltd, Zhuhai, China) and ELISA(c) (Tian Tech, Beijing Tianzhitai Biotechnology Co., LTD, Beijing, China), the above three commercial ELISA kits are recommended for us use in multi-species. The experimental procedure was performed according to each of the manufacturer's instructions. Briefly, the samples were added to the 96-well plate with a coating antigen, after 30 min of incubation at 37 °C, the plate was washed and enzyme markers added. The sample was then incubated for another 30 min at 37 °C, the plate was washed and the TMB chromogenic enzyme substrate was added. After 30 min of incubation at 37 °C, the stop solution was added, the OD_{450} was read using MultiskanTM Microplate Absorbance Reader (Thermo Scientific, Singapore). According to the criterion of the results of these commercial ELISA kits, samples were either positive, negative or in the "grey zone" for doubtful results (Table 1).

2.3. Data analysis

All statistical analyses were conducted using SPSS version 24.0. The McNemar Chi-square test was used to analyze the difference in agreement between MAT and the other different tests ($P < 0.05$ was considered significant difference). The degree of agreement among the three methods was evaluated by Cohen kappa coefficient statistics (κ), the values of κ were interpreted as follows: 0.0–0.20 = slight agreement; 0.21–0.40 = fair agreement; 0.41–0.60 = moderate agreement; 0.61–0.80 = substantial agreement; 0.81–0.1 = almost perfect agreement. The sensitivity, specificity, and area under curve (AUC) of all the ELISA tests were evaluated in comparison with the MAT, which was determined using the receiver operating characteristic (ROC).

3. Results

3.1. Results of different methods

In accordance with the cut-off titer provided by the commercial kits, of the 100 samples randomly selected from giant pandas examined for antibodies to *T. gondii*, eighty-four of the 100 samples had MAT titers of 25 (8), 50 (24), 100 (6) and 200 (46). Nine of 100 samples had IHA titers of 4 (3), 16 (2) and 256 (4) (Table 2). The seroprevalence were 84.0%

Table 1
The summary information of three commercial ELISA kits.

Serological Test	Commercial kits	Conversion formula	Interpretation (Criterion)	
ELISA (a)	ID.Screen IDVET, 310, rue Louis Pasteur, Grabels, France	$\frac{OD_S - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100\%$	Positive	S/P% ≥ 50%
			Doubtful	40% < S/P% < 50%
			Negative	S/P% ≤ 40%
ELISA (b)	Haitai Biological Pharmaceuticals Co., Ltd, Zhuhai, China	None	Positive	$OD_S > OD_C \times 1.1$
			Doubtful	$OD_C \times 0.9 \leq OD_S \leq OD_C \times 1.1$
			Negative	$OD_S < OD_C \times 0.9$
ELISA (c)	Tian Tech Beijing Tianzhitai Biotechnology Co., LTD, Beijing, China	$\frac{OD_S}{OD_C}$	Positive	S/P% ≥ 1.0
			Negative	S/P% < 1.0

OD: optical density; S: sample; NC: negative control; PC: positive control; C: control sample.

Table 2
Comparison of antibody titers to *T. gondii* of the IHA and MAT tests.

IHA titer	MAT titer					Total
	<25	25	50	100	200	
<4	16	6	24	6	39	91
4	–	–	–	–	3	3
16	–	2	–	–	–	2
64	–	–	–	–	–	0
256	–	–	–	–	4	4
Total	16	8	24	6	46	100

(95% confidence interval (CI): 76.7–91.3) by MAT, 9.0% (95% CI: 3.3–14.7) by IHA; 87.0% (95% CI: 80.3–93.7), 87.0% (95% CI: 80.3–93.7) and 84.0% (95% CI: 76.7–91.3) were sero-positive tested by ELISA (a), ELISA (b) and ELISA (c), respectively (Table 3).

In order to compare these serologic tests, MAT was considered as the reference test in this study. The 75 discordant results between the two methods were negative by IHA and positive by MAT, which resulted in 89.3% false-negative results by IHA (Table 3). On the contrary, none (0%) of the results were interpreted as false-positive by IHA. According to the criterion of the results of these commercial ELISA (a) and ELISA (b), there were four and two samples as doubtful results in ELISA (a) and ELISA (b), respectively. After excluding these, the one discordant result was negative by ELISA(a) and positive by MAT, which resulted in 1.2% false-negative results by ELISA(a); The two discordant results were negative by ELISA(b) and positive by MAT, which resulted in 2.4% false-negative results by ELISA(b); The four discordant results were negative by ELISA(c) and positive by MAT, which resulted in 4.8% false-negative results by ELISA(c). On the contrary, there were 42.9%, 43.8% and 25.0% false-positive rates in ELISA(a), ELISA(b) and ELISA(c), respectively.

3.2. Evaluation of detection methods

The degree of agreement between MAT and the other tests were calculated using the Cohen kappa coefficient (κ), the κ of IHA showed a slight agreement (κ = 0.037, 95% CI: 0.008–0.066); the κ of ELISA (b) showed a moderate agreement (κ = 0.563, 95% CI: 0.336–0.790); the κ of ELISA (a) and ELISA (c) showed substantial agreement (κ = 0.637, 95% CI: 0.336–0.790; κ = 0.702, 95% CI: 0.508–0.896). The κ analysis demonstrated that the MAT and ELISA tests had a higher degree of agreement in detecting *T. gondii* infection in giant pandas. There was no significant difference between MAT and the three ELISA tests ($P > 0.05$). However, there was a highly significant difference between the MAT and IHA tests ($P < 0.001$) (Table 4).

3.3. ROC analysis

Using MAT as the control, ROC analysis was carried out using different ELISA methods (Fig. 1). The AUC value represents the accuracy of the different tests, and the results showed that the AUC was 0.861 (95% CI: 0.712–1.000) in ELISA (a), 0.894 (95% CI: 0.791–0.997) in ELISA (b), and 0.902 (95% CI: 0.799–1.000) in ELISA (c), which revealed the optimum accuracy is ELISA (c). Sensitivity and specificity values were also assessed, the relative sensitivity of the ELISA (a, b, c) was 89.0%, 91.5% and 95.1%, respectively. Moreover, the relative specificity of the ELISA (a, b, c) was 86.7%, 80.0% and 80.0%, respectively (Table 4).

Table 3
Cross-classification, sero-prevalence and error rate of three serological tests.

	MAT	IHA		ELISA(a)		ELISA(b)		ELISA(c)	
		+	–	+	–	+	–	+	–
Cross-classification	+	9	75	81	1	80	2	80	4
	–	0	16	6	8	7	9	4	12
False negative rate (%)		89.3		1.2		2.4		4.8	
False positive rate (%)		0.0		42.9		43.8		25.0	
Sero-prevalence (%)	84.0	9.0		87.0		87.0		84.0	
95% CI	76.7–91.3	3.3–14.7		80.3–93.7		80.3–93.7		76.7–91.3	

Table 4
Comparison of diagnostic performance of IHA and ELISA kits base on MAT.

Serological test	McNemar		κ (95%CI)	Sensitivity (%)	Specificity (%)
	χ^2	*P*			
IHA	1.884	0.000**	0.037 (0.008–0.066)	–	–
ELISA(a)	44.019	0.125	0.637 (0.404–0.870)	89.0	86.7
ELISA(b)	38.904	0.180	0.563 (0.508–0.896)	91.5	80.0
ELISA(c)	49.337	1.000	0.702 (0.336–0.790)	95.1	80.0

Note:**$P < 0.01$, The difference is highly significant.
*$0.01 < P < 0.05$, difference significant.
$P > 0.05$, no significant difference.
$\kappa \leq 0.20$, The degree of agreement between two methods is slight.
$0.21 < \kappa \leq 0.40$, The degree of agreement between two methods is fair.
$0.41 < \kappa \leq 0.60$, The degree of agreement between two methods is moderate.
$0.61 < \kappa \leq 0.80$, The degree of agreement between two methods is substantial.
$0.81 < \kappa \leq 1$, The degree of agreement between two methods is almost perfect.

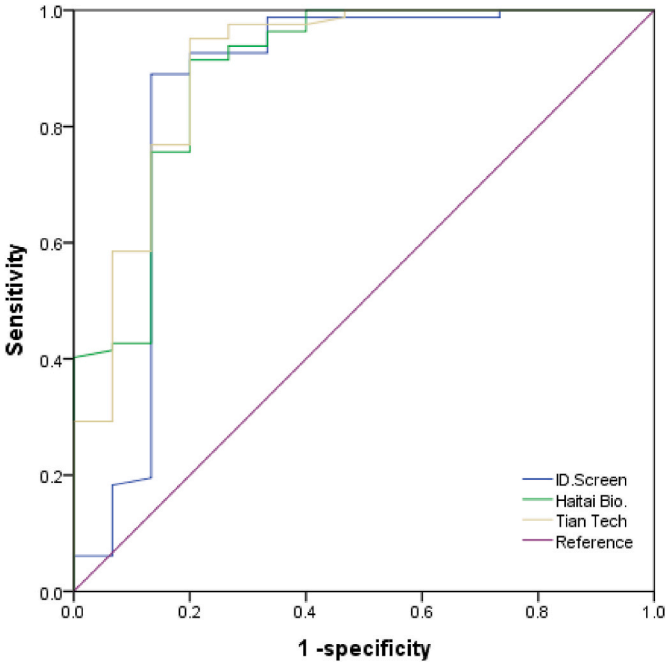

Fig. 1. Receiver operating characteristics (ROC) analysis of the ELISA.
ROC analysis shows an area under the curve (AUC) of 0.861 (95% CI: 0.712–1.000) for ELISA (a), 0.894 (95% CI: 0.791–0.997) for ELISA (b), and 0.902 (95% CI: 0.799–1.000) for ELISA (c).

4. Discussion

The zoonotic parasite *T. gondii* has a global distribution, the diagnosis of *T. gondii* infection is crucial for the surveillance, prevention and control of toxoplasmosis. Traditional approaches for the toxoplasmosis diagnosis include etiological, immunological and imaging techniques (Liu et al., 2015). Due to *T. gondii* infection usually showing no or

non-specific clinical symptoms in most individuals, serologic diagnosis is routinely used to determine the immune status in regards to *T. gondii* infection (Jenum and Stray-Pedersen, 1998). It is a difficult challenge to estimate the sero-prevalence of *T. gondii* in wildlife since there are no species-specific commercial kits. It is worth noting that comparing the performance of different tests in different host species is useful, as it allows evaluation and comparison of the results. Therefore, it is necessary to compare the performance of different tests and screen out suitable serological tests for giant pandas.

MAT is the most commonly used method for detecting antibodies to *T. gondii* in wildlife (Dubey, 2022), owing to its high sensitivity in several host species, it is also simple to perform, does not require special equipment and is available commercially, and above all, it is not species-specific and is available for use in wildlife. Moreover, MAT is widely described as the reference test when validating other serological tests, such as IHA and ELISA. Furthermore, MAT is considered specific and does not cause cross reaction with antigens of other microbes (Dubey et al., 2020). Thus, in this study, MAT was used as a reference test to compare with other serological tests and all positive samples were initially screened by MAT. However, the interpretation of MAT results is subjective, and it is recommended that MAT be performed with other serological tests for better accuracy of detection of *T.gondii* antibody of giant pandas (Hill et al., 2006).

The indirect hemagglutination assay (IHA) kit is simple, rapid, not species-specific, and is commercially available and commonly used in veterinary diagnosis for the detection of *T. gondii* antibodies. Thus, IHA is recommended for mass screening in epidemiologic surveys in some animals. However, it has poor repeatability and is unstable to sensitized red cells, limiting its application for some species (Lappin and Powell, 1991). In this study, IHA showed a slight agreement ($\kappa = 0.037$) and a highly significant difference ($P < 0.001$) compared with MAT. The results indicated the coincidence between IHA and MAT of *T. gondii* detection was low in the serum of giant pandas. Moreover, there was a high (89.3%) false-negative rate by IHA. The occurrence of false-negative and low coincidence results in IHA may be influenced by several factors, such as the analytical sensitivity of the tests, different types of antigens, or different cut-off (Dubey and Thulliez, 1989). Previous reports also showed that IHA was found insensitive in detecting antibodies to *T. gondii* in other species (Dubey, 2022). Lappin and Powell (1991) reported that IHA kits did not adequately detect *T. gondii*-specific IgM in feline samples. Fernandes et al. (2019) reported that the commercially available IHA and Latex agglutination test (LAT) are less sensitive and less specific compared with MAT. In addition, a previous study on serological investigation of *T. gondii* of giant pandas showed low sero-positivity by IHA test (Zhong et al., 2014). In summary, the results of this study suggested that IHA is not suitable for the detection of *T. gondii* infection of giant pandas.

The ELISA test has been widely used in clinical and epidemiological surveys for screening of *T.gondii* infection. It can be semi-automated, easy to perform, convenient for large-scale epidemiology surveys, and the results can be read objectively (Zhu et al., 2012). However, ELISA needs special equipment and the specificity and sensitivity depend on the antigen used (Dubey, 2022). In this study, three different multi-species commercial ELISA kits were selected to detect *T.gondii* IgG antibodies of giant pandas, and MAT was used as the reference test to compare. The results showed that sero-positivity of *T. gondii* by MAT (84.0%) was the same as ELISA (c) and slightly lower than ELISA (a) and ELISA (b) (both 87.0%); In the three ELISA tests, ELISA (c) had the highest κ value ($\kappa = 0.702$), which the degree of agreement is substantial with MAT, and ELISA (c) also had the highest sensitivity (95.1%), ELISA (a) had the highest specificity (86.7%). These results demonstrated that all the three ELISA kits have potential use for the detection of *T.gondii* antibodies of giant panda, meanwhile, the selection of specific ELISA kits will be also be based on price, availability and other factors. These results were consistent with a previous study in domestic pigs (Gamble et al., 2005). Previous investigations also showed that MAT and ELISA had good agreement for detection of *T. gondii* antibodies in both domestic animals (Lappin and Powell, 1991) and wildlife (Sharma et al., 2019).

5. Conclusion

Overall, the agreement of MAT with ELISA and IHA tests for detecting *T. gondii* antibodies of giant pandas were compared in this study, and the results showed that MAT was the recommended method for primary screening for the diagnosis of *T. gondii* antibodies in giant pandas, then verified by ELISA test. The criteria of serological antibody detection of *T. gondii* for giant pandas by testing kits is as follow, a sample was classified as positive, if the results of both the two tests showed positive, otherwise, it was negative, if the two tests showed different results, the sample needed further verification. Due to the rarity of the giant panda, no species-specific kits for IgM and IgA antibodies have been developed. Therefore, it is necessary to develop a species-specific kit for detecting *T. gondii* IgM and IgA antibodies of giant panda for diagnosis of acute infection in the future.

Declaration of competing interest

The authors declare that there are no conflicts of interest regarding this research.

Acknowledgements

We sincerely thank the veterinary staff and keepers of the Chengdu Research Base of Giant Panda Breeding for their participation in the sample collection work, James Edward Ayala for reviewing the manuscript. This research was supported by Research Project of the Science and Technology Department of Sichuan Province (2020YJ0489), Chengdu Research Base of Giant Panda Breeding (2021CPB-B11, 2021CPB-B08) and National Forestry and Grassland Administration and Sichuan Provincia Finance Department (project title: study on main epidemiological investigation and prevention of giant panda).

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