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MIC17A is a novel diagnostic marker for feline toxoplasmosis

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Abstract

Toxoplasma gondii is a widespread parasitic pathogen that infect humans and all warm-blooded animals, causing abortion and stillbirth in pregnant women and animals, as well as life threatening toxoplasmosis in immune compromised individuals. Felines are the only definitive hosts of *Toxoplasma* and oocysts shed by infected felines are the major source of infection for humans and other animals. Given the critical role of felines for *T. gondii* transmission, control of feline toxoplasmosis has significant impacts on reducing the overall prevalence of animal and human toxoplasmosis. However, reliable diagnosis of feline toxoplasmosis is still challenging. In this study, we found that the putative micronemal protein 17A (MIC17A) that was abundantly expressed in *Toxoplasma* merozoites is a good diagnostic marker for serological diagnosis of *Toxoplasma* infection in felines. *T. gondii* encodes four paralogs of MIC17A in total and the expression of three of them is drastically upregulated in merozoites than in tachyzoites. In contrast, when proteins like GRA1 and MIC3 that are more abundantly expressed in tachyzoites than in merozoites were used as diagnostic antigens to test feline toxoplasmosis, they reacted with *Toxoplasma* specific IgG antibodies poorly. Taken together, these results suggest that merozoite antigens are better suited for the diagnosis of feline toxoplasmosis than antigens that are highly expressed at tachyzoite or bradyzoite stages.

Keywords: Cat, *Toxoplasma*, Diagnosis, Merozoites, indirect ELISA

Introduction

T. gondii is an obligate intracellular parasite infecting a wide variety of animals, including humans, birds, and mammals (Hill et al. 2005). It is estimated that up to one-third of the world's human population may be infected with *T. gondii* (Montoya and Liesenfeld 2004; Dubey 2008). The vast majority of these infections are at the chronic stage where the infected individuals do not show obvious symptoms (Desmonts and Couvreur 1974). Nonetheless, the parasites are living in the central nerve system and muscles and replicating at a very slow rate (Dubey et al. 1998). Once the immune functions are compromised, such as those with HIV infection or organ transplantation, chronic infections may be reactivated to acute infections and cause serious complications (Wang, Wang et al. 2017). In US, *T. gondii* is ranked second in terms of the annual burden of disease caused by all major foodborne pathogens (Batz, Hoffmann et al. 2012). It causes more than 300 deaths and 3 billion dollars of illness cost each year (Hoffmann, Batz et al. 2012). In addition, vertical transmission during pregnancy is another major risk of *Toxoplasma* infection, which can cause abortion and still birth of pregnant women and animals (Song et al. 2005). In some regions like Ireland and Northern Ireland, toxoplasmosis is the No.1 cause of abortion in ewes, accounting for up to 24% of all abortion cases (Innes et al. 2009). As such, *T. gondii* is a zoonotic pathogen of great medical and veterinary significance.

One key reason for the high prevalence of *T. gondii* is its complex life cycle and multiple routes of transmission. A complete life cycle involves intermediate hosts and

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definitive hosts (Miller et al. 1972). While essentially all warm-blooded animals can serve as intermediate hosts of *T. gondii*, felines are the only definitive hosts. *T. gondii* reproduces asexually in the intermediate host and exists in the forms of tachyzoites and bradyzoites, which are responsible for acute and chronic infections respectively. One unique aspect of *Toxoplasma* is that it can be transmitted between intermediate hosts, without the need of definitive hosts to complete the life cycle. This is due to its ability to interconvert between tachyzoites and bradyzoites (Skariah et al. 2010). For instance, one major route of human infection is through the ingestion of undercooked meat (pork and lamb etc.) that contains *Toxoplasma* bradyzoites (Cook et al. 2000). In addition, transmission of *Toxoplasma* between intermediate hosts through predation is also very common among wild and domestic animals (Dubey et al. 1998).

Felines as the only definitive hosts of *Toxoplasma* have a major and unique role during its transmission. *Toxoplasma* undergoes sexual replication in intestinal epithelial cells of felines to produce oocysts, which are highly infectious and environmentally resistant after sporulation. One infected feline can excrete millions of oocysts in a short period of time (Dubey 2005). As such, oocyst contaminated water and fresh produce are a main source of infection for humans and animals. Oocyst production is a very complicated process and very little is known about the underlying biology. The most efficient way of oocyst shedding is when felines are infected with bradyzoites (Dubey 1998). After infection, the parasites convert to merozoites and proliferate rapidly in intestinal epithelial cells through endopolygeny. Then, some merozoites are differentiated into male and female gametes, which then fuse to produce oocysts. Mature oocysts are released from epithelial cells and shed to the environments along with feline feces (Black and Boothroyd 2000). Under appropriate humidity and temperature conditions in the environment, oocysts quickly sporulate and become infectious to other animals. However, the detailed mechanisms that govern each of the developmental steps during oocyst formation are poorly understood. Transcriptomic analyses have clearly shown that gene expression in merozoites and during sexual reproduction is significantly differently than that in tachyzoites or bradyzoites (Behnke et al. 2014). On the other hand, felines may also act as intermediate hosts for *T. gondii*. But the relationship of this role of felines has not been well connected to their role as definitive hosts.

Healthy felines infected with *Toxoplasma* often do not show obvious symptoms and the diagnosis of feline toxoplasmosis is still challenging (Dubey 1995). In the literature, many different methods have been used to assess the serologic prevalence of *Toxoplasma* infection in felines. These include indirect fluorescent tests, indirect hemagglutination, modified agglutination tests (MAT), Enzyme-Linked Immunosorbent Assay (ELISA) and others (Liesenfeld et al. 1996). Using these tests, the prevalence of *Toxoplasma* infection in felines varied from 0 to 100%, depending on the geographic region and the source of the felines (stray felines and home owned pet felines etc.) (Dubey et al. 2020). One common problem of these studies is that the accuracy of the corresponding methods is often a concern. Taking ELISA for example, many different antigens have been used but how well they agreed with each other in terms of the testing results is not known. The majority of these methods use either total soluble antigens (TSA) of tachyzoites or recombinant proteins as diagnostic antigens (Choi et al. 1992). For recombinant antigens, *Toxoplasma* surface antigens or secretory proteins from specialized organelles like dense granules, rhoptries, micronemes are preferred choices, due to their high levels of expression and immunoreactivity. While native TSA seems to be the most reliable antigen for diagnostic purposes, to use it is not as convenient as recombinant antigens because its production involves culture of live parasites that are of safety concerns. In this study, we have tried to find a recombinant antigen that can replace TSA for the diagnosis of feline toxoplasmosis. To our surprise, the proteins like GRA1, GRA7 and MIC3 that are commonly used for the diagnosis of *Toxoplasma* infection in intermediate hosts (Pietkiewicz et al. 2007; Jiang et al. 2008), did not perform well for the diagnosis of feline toxoplasmosis. On the other hand, we found that the MIC17A protein highly expressed in the merozoite stage of *Toxoplasma* was a suitable marker for the diagnosis of toxoplasmosis in felines.

Results

Feasibility of GRA1, MIC3, BAG1, GRA7, and GRA10 as antigens for the diagnosis of feline toxoplasmosis

To find a recombinant protein suitable as a diagnostic antigen for feline toxoplasmosis, we first selected five antigens from commonly used iELISA methods that are used to detect toxoplasmosis in intermediate hosts like sheep and swine. These include GRA1, MIC3, BAG1, GRA7 and GRA10. The five recombinant proteins were expressed in *E. coli* and purified to nearly homogeneity, using the methods described previously. Then, 2 μg/mL proteins were coated into ELISA plate wells (100 μL per well). To test whether these proteins could be used for the diagnosis of cat diagnosis, six cat serum samples that were confirmed to be positive for *T. gondii* IgG by MAT and TSA-iELISA were examined using the above protein coated ELISA plates. The results showed that the S/N (OD_{630} of samples / OD_{630} of negative control) values

were very low when using these five proteins as diagnostic antigens (Table 1). In contrast, the S/N numbers from TSA-iELISA were close to or above 10, much higher than those obtained from GRA1, MIC3, BAG1, GRA7, or GRA10 based ELISA. These results suggest that these antigens may not be good for serological diagnosis of feline toxoplasmosis.

Looking for novel diagnostic markers for feline toxoplasmosis

The above five antigens were frequently used in the lab as diagnostic markers to check *Toxoplasma* infection in intermediate hosts. They are abundantly expressed either at the tachyzoite or the bradyzoite stage. Since the above results showed that they were not suitable for the detection of feline toxoplasmosis, we sought to identify other antigens that could service this purpose. We speculated that when *T. gondii* infects the definitive hosts, cats, tachyzoites or bradyzoites might not be the main parasite forms that activate immune responses and stimulate antibody production. On the other hand, merozoites are the critical stage for *T. gondii* to enter sexual reproduction in the intestinal epithelial cells of cats. In this regard, we hypothesized that proteins that are highly expressed in merozoites might be useful for the diagnosis of cat toxoplasmosis. To this end, we used the transcriptomic data available in ToxoDB (https://toxodb.org/toxo/app) and looked for proteins that are highly expressed in merozoites and predicted to be surface or secretory antigens. After comparison, we selected the protein MIC17A (TGME49_200250) to test our hypothesis.

MIC17A was predicted to be a secretory protein localized to the secretory organelle micronemes. MIC17A has a plasminogen apple nematode (PAN) domain that is commonly found in MIC proteins. Transcriptomic data from ToxoDB show that MIC17A is highly expressed in merozoites, reaching an expression percentile of 99.99% with a transcripts per million (TPM) value of more than 10,000. In addition, *T. gondii* encodes four paralogs of MIC17A, three of which (MIC17A, MIC17B and MIC17D) are also significantly upregulated in merozoites than in tachyzoites. Expression of MIC17C was low in both tachyzoites and merozoites (Fig. 1A). Sequence alignment of the four MIC17 paralogs showed that they are highly similar to each other (Fig. 1B). As such, the antibodies generated from one paralog are expected to react with all four proteins. MIC17A sequence was also used in a Blast search to see if there are homologous sequences in other organisms. The results show that highly similar sequences could be found in *Neospora caninum*, *Hammondia hammondi* and *Besnoitia besnoiti*, which are closely related to *T. gondii*. Although other coccidian parasites like *Cystoisospora*, *Eimeria* and *Cyclospora* may also encode MIC17 homologs, the sequence identity with *Toxoplasma* proteins is below 30% and the antibodies generated are less likely to cross-react.

MIC17A reacted strongly with *T. gondii* positive cat sera

To test whether MIC17A can be used for the diagnosis of *Toxoplasma* infection in cats, it was expressed recombinantly in *E. coli* and tested with cat serum samples that are confirmed to be positive for *Toxoplasma* specific IgG. First, the coding sequence of MIC17A was cloned from the cDNA of the type II strain ME49 and cloned into the pET-28a to express it as a 6 x His fusion protein. The resulting pET-28a-MIC17A plasmid was introduced into BL21 (DE3) cells and expression of the His-MIC17A fusion protein was induced by IPTG. Subsequently the bacterial cells were lysed and expression of proteins were examined by SDS-PAGE. The results showed that the recombinant His-MIC17A was mainly expressed in an insoluble form in the inclusion bodies (Fig. 2A). To purify the recombinant protein, pelleted inclusion bodies were first denatured with 0.3% sarkosyl and then renatured in PBS. Then, the solubilized proteins were loaded to a nickel column for affinity purification. Imidazole eluted fractions were analyzed by SDS-PAGE and pure His-MIC17A fractions were collected and dialyzed in PBS before use (Fig. 2B). To test whether the purified recombinant His-MIC17A could react with the IgG antibodies in cat sera, it was subjected to Western blot analyses using *Toxoplasma* positive and negative sera respectively.

Table 1 Indirect ELISA test results of cat sera using indicated antigens

	GRA1	GRA10	GRA7	BAG1	MIC3	TSA
Sample #1	2.5 [a]	2.01	3.95	1.9	3	12.56
Sample #2	3.3	3.2	4.19	1.67	4.07	12.64
Sample #3	1.69	2.39	2.66	1.55	3.29	10.26
Sample #4	2.7	3.43	3.55	1.43	3.64	10.98
Sample #5	3.4	4.3	4.11	1.27	4.14	9.59
Sample #6	1.32	1.2	1.77	0.96	1.57	11.3

[a] S/N (OD_{630} of sample / OD_{630} of negative control) value of each sample

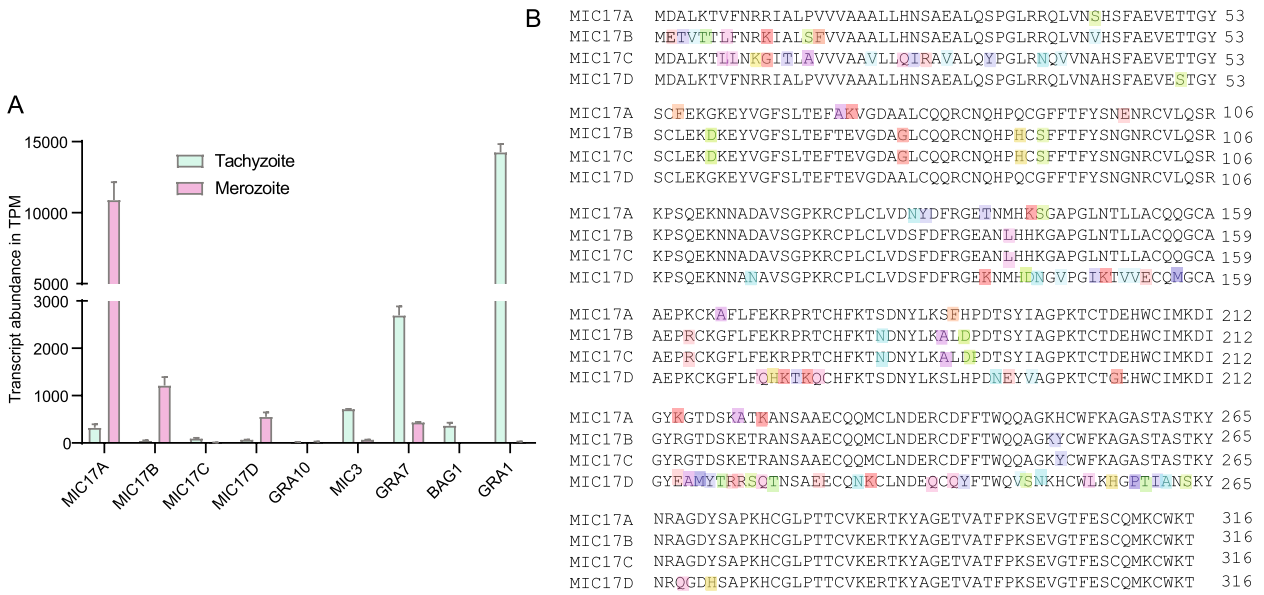

Fig. 1 Expression patterns and sequence alignment of MIC17A paralogs. **A** Expression levels of indicated genes at the tachyzoite and merozoite stages respectively. Transcript level is shown as transcripts per million (TPM). Data were extracted from ToxoDB (data set *Tachyzoite and merozoite transcriptomes (Hehl* et al.*)*). **B** Sequence alignment of four MIC17A paralogs encoded by *T. gondii*, which was done by Clustal X2. Gene ID for these paralogs: MIC17A: TGME49_200250; MIC17B: TGME49_200240; MIC17C: TGME49_200230; MIC17D: TGME49_200270

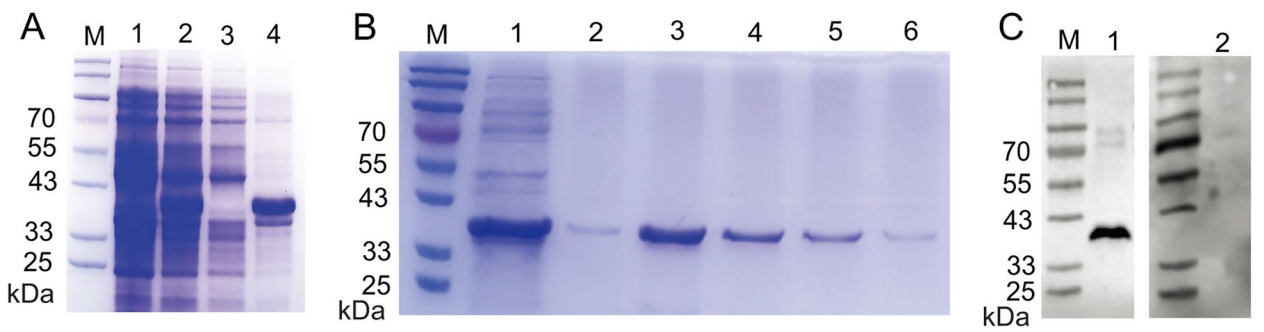

Fig. 2 Expression and purification of recombinant MIC17A. **A** MIC17A was mainly expressed in the inclusion bodies. M: marker; 1: total lysate from culture without IPTG induction; 2: total lysate of IPTG induced culture; 3: supernatant faction of IPTG induced culture; 4: pellet faction of IPTG induced culture. **B** Purification of MIC17A by a nickel column. M: marker; 1: flow-through fraction after loading denatured and renatured pellets to the nickel column; 2–6: eluted fractions after washing with different concentrations of imidazole. 2: 0%, 3–4: 5%, 5–6: 10%. **C** Western blotting checking the reactivity of MIC17A to cat sera. M: marker; 1: incubated with *Toxoplasma* positive cat serum; 2: incubated with *Toxoplasma* negative cat serum

The results clearly showed that His-MIC17A could be easily detected with the *Toxoplasma* positive serum, but not with the negative serum (Fig. 2C), suggesting that MIC17A is able to distinguish the infection status of cats.

To further check whether MIC17A can be used as a diagnostic antigen in ELISA tests, we coated the ELISA plates with 2.25 μg/mL recombinant MIC17A and used them to test six cat serum samples (the same samples as those used in Table 1) that were confirmed to be positive for *Toxoplasma* specific IgG. The calculated S/N values are all above nine (Table 2), much higher than those obtained when using GRA1, MIC3, BAG1, GRA7,

Table 2 Indirect ELISA test results of cat sera using MIC17A as the coating antigen

	MIC17A	TSA
Sample #1	9.72 [a]	11.8
Sample #2	10.69	11.93
Sample #3	9.92	9.74
Sample #4	10.87	10.38
Sample #5	10.44	9.12
Sample #6	10.47	10.65

[a] S/N (OD_{630} of sample / OD_{630} of negative control) value of each sample

or GRA10 as diagnostic antigens (Table 1). These results further suggest that MIC17A is a feasible antigen for serological diagnosis of cat toxoplasmosis.

The results of MIC17A-based ELISA tests agree well with that of MAT and TSA based ELISA

From the above tests on a small number of serum samples, it seems like the merozoite antigen MIC17A based ELISA has a higher positive detection rate than those based on tachyzoite or bradyzoite antigens like GRA1 and BAG1. To check whether the high positive detection rate is real or false positive, we compared the MIC17A-iELISA to other tests that are frequently used for the diagnosis of *Toxoplasma* infection. First, it was compared to the TSA based iELISA, which is a widely accepted method to test *Toxoplasma* specific antibodies in host serum samples. A total of 36 samples were analyzed by both optimized MIC17A-iELISA and TSA-iELISA. The results showed that 19 tested positive and 11 were negative in both tests (Table 3). Only six samples displayed discrepancy, which tested positive in MIC17A-iELISA but negative in TSA-iELISA. Therefore, the overall agreement rate between these two methods is 83.3% ((19 + 11)/36 = 83.3%). MAT is another commonly used approach to examine the presence of *T. gondii* specific antibodies. A comparison between MIC17A-iELISA and MAT involving 106 samples found that there is a good agreement between these two methods as well. Of the samples analyzed, 24 tested positive and 67 tested negative in both tests (Table 4). The overall agreement is 85.8%. Lastly, we compared MIC17A-iELISA to a commercial kit (ID Screen Toxoplasmosis Indirect Multi-species from Innovative Diagnostics), which is an indirect ELISA test using SAG1 as the diagnostic antigen (therefore we call it SAG1-iELISA). Interestingly, of the 60 samples tested, only eight samples had the same results from both tests (four were positive and the other four were negative). Fifty-two samples that tested positive by MIC17A-iELISA tested negative by the commercial SAG1-iELISA kit (Table 5), suggesting a much lower positive detection rate of SAG1-iELISA than MIC17A-iELISA. Given the decent agreement between MIC17A-iELISA and MAT as well as TSA-ELISA, these results suggest that the low positive detection rate of SAG1-iELISA kit is likely due to the low sensitivity of this kit. SAG1 is a surface antigen highly expressed in tachyzoites, similar to GRA1 in Fig. 1A. These results are consistent with the data above showing that tachyzoite or bradyzoite antigens like GRA1, MIC3, BAG1, GRA7, and GRA10 were not ideal for the diagnosis of cat toxoplasmosis.

Some infected cats may not develop chronic infection

It is widely assumed that cats are both definitive and intermediate hosts for *T. gondii*. Once an intermediate

Table 3 Comparison between the MIC17A-iELISA and TSA-iELISA

		MIC17A-iELISA results		Overall agreement
		Positive	Negative	
TSA-iELISA results	Positive	19 [a]	0	(19 + 11)/(19 + 0 + 6 + 11) = 83.3%
	Negative	6	11	

[a] Number of serum samples in each category

Table 4 Comparison between the MIC17A-iELISA and MAT test

		MIC17A-iELISA results		Overall agreement
		Positive	Negative	
MAT test results	Positive	24	11	(24 + 67)/(24 + 11 + 4 + 67) = 85.8%
	Negative	4	67	

Table 5 Comparison between the MIC17A-iELISA and SAG1-iELISA

		MIC17A-iELISA results		Overall agreement
		Positive	Negative	
SAG1-iELISA results	Positive	4	0	(4 + 4)/(4 + 0 + 52 + 4) = 13.3%
	Negative	52	4	

host is infected with *Toxoplasma*, the parasite will stay in the host lifelong, mostly in the form of chronic infection. As such, infected cats are assumed to contain parasites in their tissues. The big discrepancy between the MIC17A-iELISA and SAG1-iELISA, as well as ELISAs using GRA1, MIC3, BAG1, GRA7 and GRA10 as diagnostic antigens, prompted us to check the underlying reasons. One possibility is that in some infected cats, the parasites may only go through coccidian development in gut epithelial cells to produce oocysts without penetrating deeper to form tachyzoites and bradyzoites, like that in *Eimeria* parasites. To check this possibility, we screened a number of cats and tested the presence of *Toxoplasma* DNA in their heart and brain tissues, which are preferred places for chronic infection establishment. Three cats (Cat 1, 2 and 6) that tested negative in MIC17A-, TSA- and SGA1-based ELISAs were found to be free of *Toxoplasma* parasites, since the PCR amplification of the *Toxoplasma* specific 529-bp repeat failed to amplify positive products (Supplementary Fig. S1 A&B). On the other hand, two cats that tested positive in the above three ELISAs did have *Toxoplasma* parasites in their tissues, as the 529-bp repeat was successfully amplified from their heart and brain tissues (Supplementary Fig. S1A). Interestingly, one cat (Cat 5) that tested positive in MIC17A- and TSA-ELISA but negative in SAG1-ELISA was found to be free of *Toxoplasma* in tissues (Supplementary Fig. S1B). These results indicate that this cat had a history of *Toxoplasma* infection but did not develop chronic infection, supporting our hypothesis that in some infected cats, *Toxoplasma* only undergoes coccidian development without forming tachyzoites or tissue cysts.

Toxoplasma infection is common in pet cats

While the MIC17A-iELISA was proved to be useful for the diagnosis of *Toxoplasma* infection in cats, it was used to estimate the prevalence of toxoplasmosis in pet cats. A total of 143 cat serum samples were collected from a local pet hospital in Wuhan city and tested by optimized MIC17A-iELISA. The overall seroprevalence is 30%. There is a slight difference in the seroprevalence rates among different types of cats. 42.8% (3/7) of Garfield cats and 35% (7/20) British shorthair cats were seropositive, whereas only 21.4% (3/14) of American Shorthair cats and 25% (4/16) Ragdoll cats tested positive (Fig. 3A). On the other hand, gender of the cats does not seem to affect seroprevalence, as 30% female cats and 30.1% male cats were tested positive (Fig. 3B).

Discussion

Felids are the only definitive hosts of *T. gondii* and they play critical roles in the transmission of *Toxoplasma* parasites (Frenkel et al. 1970). Therefore, proper control of cat toxoplasmosis is of great significance to reduce the overall prevalence in humans and animals. Unfortunately, there is still no reliable diagnostic method for the detection of feline toxoplasmosis. Previous work from our lab has shown that the total soluble antigen (TSA) prepared from tachyzoites can be used as diagnostic antigens to examine the presence of *Toxoplasma* specific IgG antibodies in cat sera. This partially solved the problem of serodiagnosis of cat toxoplasmosis. However, the production of TSA requires the culture of live parasites, which may have safety concerns. In this study, we sought to find a recombinant antigen that can replace TSA for the serodiagnosis of *Toxoplasma* infection in cats. We found that selected tachyzoite and bradyzoite antigens that are commonly used for serodiagnosis of *Toxoplasma* infection in intermediate hosts were not ideal for diagnosis of cat toxoplasmosis. In contrast, the putative micronemal protein MIC17A that is highly induced in merozoites is as good as TSA for the serodiagnosis of cat toxoplasmosis (Table 3). This study not only discovered a novel diagnostic antigen for cat toxoplasmosis, but also offered

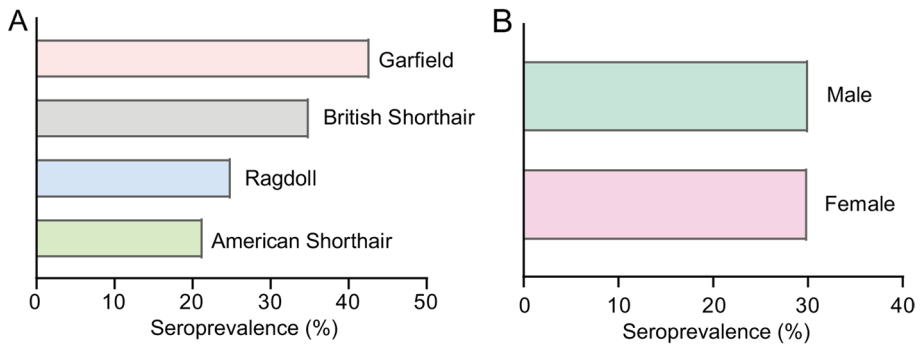

Fig. 3 Prevalence of *Toxoplasma* infection in different types of cats (**A**) or different gender of cats (**B**), as determined by MIC17A-based indirect ELISA

important insights into the complex biology of *Toxoplasma* parasites in cats.

In the past, the vast majority of diagnostic antigen screens focus on infections in intermediate hosts. In addition, it is generally accepted that cats are both definitive and intermediate hosts of *T. gondii*. As such, it is thought that the antigens used for diagnosing *Toxoplasma* infection in intermediate hosts may also work for the diagnosis of cat infection. For example, the commercial kit ID Screen Toxoplasmosis Indirect Multi-species ELISA is claimed to be able to detect anti-*Toxoplasma* antibodies in multiple species, including cats. This kit uses the tachyzoite surface antigen SAG1 as diagnostic antigen. Compared with our MIC17A-iELISA, the SAG1-iELISA had a much lower positive detection rate (Table 5). A significant number of samples that tested positive in MIC17A-iELISA were diagnosed as negative in SAG1-iELISA. On the other hand, the results of MIC17A-iELISA agreed well with that of TSA-iELISA or MAT. Together, these results suggest that SAG1, just like other tachyzoite or bradyzoite antigens tested in this study (GRA1, MIC3, BAG1, GRA7 and GRA10), is not a suitable antigen for serodiagnosis of cat toxoplasmosis.

While tachyzoite and bradyzoite proteins like SAG1 and GRA1 are promising antigens for serodiagnosis of *Toxoplasma* infection in intermediate hosts (Burg et al. 1988; Cesbron-Delauw et al. 1989), their incompetence in the diagnosis of cat toxoplasmosis calls into question regarding the growth and development of *Toxoplasma* in cats. Because as mentioned above, cats are also intermediate hosts of *T. gondii*. Our results have provided one possible explanation for this conundrum. We believe that a portion of infected cats do serve as both intermediate and definitive hosts, meaning that they support sexual reproduction of the parasites to produce oocysts, meanwhile they contain tachyzoites and/or bradyzoites in diverse tissues. On the other hand, certain cats may only serve as definitive hosts during the infection and they do not support tachyzoites or bradyzoites development in other tissues, similar to corresponding hosts infected with *Eimeria* and *Cryptosporidium* parasites. We have found one cat, whose serum was tested to be positive in MIC17A-iELISA and TSA-iELISA but negative in SAG1-iELISA. When PCR amplification of the 529-bp repeat was used to assess the presence of *Toxoplasma* parasites in its brain and heart tissues, it was found that this cat did not contain parasites in these tissues. This is one case that supports the above hypothesis. This cat might have only supported the coccidian development of *Toxoplasma* (therefore it was MIC17A-iELISA and TSA-iELISA positive), but not tachyzoites or bradyzoites (SAG1-iELISA negative). Together, these results also suggest that merozoite antigens are likely major drivers of cat immune responses to produce antibodies during *Toxoplasma* infection. As such, antigens like MIC17A that are highly expressed in merozoites are promising antigens for serodiagnosis of *Toxoplasma* infection in cats.

Conclusions

We found that the MIC17A protein that is abundantly expressed in *Toxoplasma* merozoites is a promising antigen for serodiagnosis of *Toxoplasma* infection in cats. MIC17A-iELISA had much higher positive detection rates than the ELISA tests using tachyzoite and bradyzoite antigens like SAG1 and GRA1. *Toxoplasma* parasites may undergo coccidian development in some infected cats to produce oocysts, without penetrating further to form tachyzoites and bradyzoites. This may explain why MIC17A is better suited for the serodiagnosis of cat toxoplasmosis than tachyzoite or bradyzoite antigens.

Methods

Parasite strains and source of serum samples

T. gondii strain RH and ME49 were propagated in HFF cells, which were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 mg/mL penicillin-streptomycin and 5 mM L-glutamine. Cats for experimental purposes were purchased from a cat breeding facility in Shandong Province of China. *T. gondii* positive and negative control sera were verified by TSA-iELISA and MAT before use. All other serum samples were collected from pet hospitals in Wuhan, China. All animal experiments were approved by the Scientific Ethic Committee of Huazhong Agricultural University (permit #: HZAUCA-2022-0007).

Construction of the pET-28a-MIC17A plasmid and purification of the recombinant MIC17A protein

The coding sequence of MIC17A was amplified from the cDNA prepared from the type II strain ME49, using the following primers: MIC17A-F: 5′-AGCAAATGG GTCGCGGATCCGGGCTCCGGAGACAGCTAGTC; MIC17A-R: 5′-TCCTTTCGGGCTTTGTTTTAGCAT GTGATATCGCCTGCTT. Meanwhile the plasmid backbone was amplified from pET-28a using primers vet-F: 5′-AACAAAGCCCGAAAGGAAGC and vec-R: 5′-GGA TCCGCGACCCATTTGC. Then the MIC17A fragment was ligated with the plasmid backbone using the ClonExpress II Kit *(Vazyme, China)*. The resulting pET-28a-MIC17A plasmid was sequenced before use. To express the recombinant MIC17A protein, the pET-28a-MIC17A plasmid was transformed into *E. coli* competent cells BL21 (DE3). IPTG at the final concentration of 1.0 mM was used to induce MIC17A expression. To purify MIC17A, induced bacterial cultures were collected and lysed on a French press. The lysates were centrifugated

at 14000 rpm for 10 min and the pellets were collected, denatured in 0.3% sarkosyl *(Solarbio, China)* and then renatured through dialysis in PBS. The renatured proteins were then loaded to a nickel column for purification and MIC17A protein was eluted with different concentrations of imidazole. Eluted MIC17A was dialyzed again in PBS, quantified by a BCA Protein Assay Kit *(Beyotime, China)*, and then stored at −80 °C for further use.

Western blotting

Purified MIC17A protein samples were separated on a 12% SDS-PAGE gel and then transferred to nitrocellulose membrane. Then the membrane was probed with *Toxoplasma* positive or negative cat sera that were validated by MAT and TSA-iELISA. HRP conjugated rabbit anti-feline IgG *(Biolab, China)* was used to detect primary antibodies and the blots were developed using ECL chemiluminescence reagents *(Beyotime, China)* and imaged with a chemiluminescence imager *(Tanon, China)*, as previously described (Gross et al. 1992).

MAT test

Freshly egressed tachyzoites of the type I strain RH were purified and fixed with 6.7% formaldehyde solution, resuspended in alkaline buffer to a final concentration of 2×10^7 mL tachyzoites, to prepare the MAT antigen stock as previously described (Desmonts and Remington 1980). The MAT tests were performed in U-shaped 96-well plates using the methods described before (Dubey and Desmonts 1987). The serum samples were tested at serial 2-fold dilutions starting at 1:25. Controls using validated positive and negative cat sera were included in each plate.

Elisa

His-SUMO-GRA1, His-SUMO-GRA10, GST-MIC3, His-BAG1, GST-GRA7, His-MIC17A or TSA were coated into the microtiter ELISA plates (100 µL per well, concentration of proteins was 2 µg/mL except His-MIC17A, which required 2.25 µg/mL) and incubated at 4 °C overnight. Then the plates were blocked with 1% BSA and incubated with the serum samples to be tested (Dubey 2009). HRP conjugated rabbit anti-feline IgG (Biolab, China) was used as the secondary antibody. After washing off the unbound secondary antibody, TMB substrate solution *(Solarbio, China)* was added (100 µL per well) and reacted at room temperature for 10 min in the dark. Then 0.25% hydrofluoric acid (50 µL per well) was added to each well to stop the reactions. Finally, the OD_{630} value of each well was measured using a cytation 5 microplate reader *(BioTek, USA)*. Validated positive and negative sera were used as controls and the S/N value (OD_{630} of a given sample / OD_{630} of the negative control) was used to determine the infection status of a given sample.

Abbreviations

MAT: Modified agglutination test; MIC17A: micronemal protein 17A; GRA1: dense granule protein 1; GRA10: dense granule protein 10; BAG1: bradyzoite antigen 1; HRP: horseradish peroxidase; ELISA: Enzyme Linked Immunosorbent Assay.

Supplementary Information

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Additional file 1: Fig. S1. PCR amplification of tissue samples from cats for the presence of *Toxoplasma* specific DNA. **A**: Heart and brain tissues from cats that were tested positive or negative by MIC17A, TSA or SAG1 based ELISAs were examined by PCR amplification of the 529-bp repeat fragment. M: DNA marker, −/+ control denote positive and negative controls. **B**, PCR amplification (529-bp repeat) of tissues isolated from SAG1 negative, but MIC17A and TSA positive or negative cats.

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Authors' contributions

JC performed the experiments, analyzed the data, and drafted the manuscript. LX, HH and XY contributed to the animal experiments and performed serological analyses. HC performed some of the key TSA-iELISA experiments and analyzed the data. BS conceived and supervised the project, analyzed the data, and revised the manuscript. All authors have read and approved the submitted version of the manuscript.

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Availability of data and materials

The *Toxoplasma* gene expression data analyzed in this study are available in ToxoDB (https://toxodb.org/toxo/app/record/dataset/DS_75742b9a0a). All other data generated in this study are included in this published article.

Declarations

Ethics approval and consent to participate

All animal experiments in this study have been approved by the Scientific Ethic Committee of Huazhong Agricultural University (permit #: HZAUCA-2022-0007). Cat sera samples were collected from a local pet hospital in Wuhan and consents from cat owners were obtained before sera collection.

Consent for publication

Not applicable.

Competing interests

A patent has been filed in China to protect the use of MIC17A in serological diagnosis of cat toxoplasmosis. An intent of collaboration with Wuhan Keqian Biology Co., Ltd. has been agreed to develop diagnostic products using MIC17A. Author Bangshen was not involved in the journal's review or decisions related to this manuscript.

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