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Toxoplasmosis in cats from a dairy-producing region in Hidalgo, Mexico

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Abstract

Considering that the ingestion of cat feces containing *Toxoplasma gondii* oocysts is a major transmission route for toxoplasmosis, this work is aimed to assess the presence of *T. gondii* in cats dwelling in dairy production facilities at the Tizayuca Agro-Industrial Complex (CAIT, by its acronym in Spanish) in Hidalgo, Mexico. Blood samples from stray and owned cats were assayed by indirect ELISA, and samples from various tissues were collected at *post-mortem* examination of positive cats for DNA extraction, PCR, histopathologic studies, and immunohistochemistry. A questionnaire was applied in each dairy production unit to estimate cat ownership. From 70 animals studied (22 stray and 48 owned), 12 (17.1 %) were seropositive (7 stray and 5 owned). No differences were found for age or sex according to the questionnaires, but ingesting raw food was a risk factor ($P < 0.01$). Of the 12 positive cats, 5 were euthanized; of these, five tissue samples from 4 cats were positive for the *B1* and 529 bp repetitive sequences by qPCR and conventional PCR. No macro- or microscopic lesions suggestive of *T. gondii* infection were found in cats.

Keywords: *Toxoplasma gondii*; Indirect ELISA; Dairy production; Isolation; Toxoplasmosis prevalence; Toxoplasmosis transmission.

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Study contribution

This study confirmed that the conditions prevailing in the CAIT are conducive to the presence and transmission of a zoonosis as serious as toxoplasmosis. Predisposing factors are concurring there, including the coexistence of stray cats with livestock and people, which enables the continuation of the epidemiological cycle and represents an important aspect that should be considered when proposing prevention and control programs, highlighting the need to have not only serological but also molecular evidence.

Introduction

Toxoplasma gondii is a protozoan of the *Apicomplexa phylum*, which includes more than five thousand species, some of them associated with patterns of clinical and subclinical disease in animals and humans. It is a unique species, although many genetic variants have been described, and some of them have been reported in Mexico.[1–3] This parasite is an important pathogen in human and veterinary medicine because of its ability to cause spontaneous abortion and congenital disease in intermediate hosts.[3–5] The parasite has been estimated to infect over one-third of the world population. Serological positivity rates vary widely in human populations, from 15 to 85 %, depending on the region and factors like age and cultural practices.[3, 6]

Felines are definitive hosts of *T. gondii*, as they carry the parasite's sexual (and asexual) stages. They can become infected by ingesting meat from prey harboring tissue cysts or oocysts from the environment. Whilst felines are usually asymptomatic, they can excrete up to ten million non-sporulated oocysts in their feces daily for 7–15 days when temperature and relative humidity increase sporulation rates, reaching an infective stage in 24–48 h after shedding, and remain infective for months. The main risk factors for *T. gondii* infection in cats are consumption of raw or undercooked meat or food leftovers, age, and hunting habits.[4, 7, 8] On the other hand, intermediate hosts are infected by contact with soil, water, grass, or crops contaminated with sporulated oocysts, or by direct consumption of cysts in raw or undercooked meat. Another transmission route occurs during the pregnancy, since tachyzoites can reach the fetus through the placenta, resulting in miscarriage or fetal damage.[6, 7]

The prevalence of cat toxoplasmosis varies widely throughout the world. For instance, prevalence rates of 17.2 % and 40.0 % were reported in stray animals from China and Iran, respectively, while prevalence rates of 25.5 % and 36.9 % were reported in owned cats and stray felines from Spain.[9–11] With respect to Mexico, several authors have reported prevalence rates of 21–63 % in cats from various Mexican states.[12–15] ELISA, histopathological examination, PCR, and direct isolation have been used to diagnose *T. gondii* infection.[2, 7, 16] Depending on the virulence of each isolate, mice can develop either an acute infection with a high tachyzoite load in 8–12 days, or a chronic infection, with low to null mortality and several tissue cysts, most often located in the central nervous system and heart.[3, 17] Diagnosis cannot rely solely on the clinical picture, since clinical signs and postmortem findings are non-specific.[3, 7]

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This is relevant in settings where cats and livestock species such as bovines live next to each other, especially where the latter are used for human consumption. Such is the case of the Tizayuca Agro-Industrial Complex (CAIT, by its acronym in Spanish), where humans, cattle, dogs, cats, and rodents live together. An epidemiological study in dairy production areas like this would provide key information about the transmission and epidemiological status of the complex. Therefore, this study was aimed to determine the presence of *T. gondii* in cats living in dairy production facilities at the CAIT in Hidalgo, Mexico.

Material and methods

This is an observational, descriptive, prospective, cross-sectional study.

Ethical statement

The Institutional Subcommittee for the Care and Use of Experimental Animals (SICUAE, for its acronym in Spanish), Graduate Section, UNAM. The protocol was approved on January 13, 2012.

Location

The CAIT is located at 2 260 meters above sea level, in the south of the state of Hidalgo, Mexico, at a latitude of 19º 51′ 25″ N and a longitude of 98º 50′ 8″ W, covering an area of 220 hectares. The climate is generally is dry and semi-cold, with rainfall in summer, the highest humidity season. The complex houses 120 dairy production (DP) units with about 16 000 cows and 6 000 replenishing calves. Each production unit was geolocated using a 76CSx global positioning system (GPS) unit (Garmin, Olathe, KS, USA).

Questionnaire

A questionnaire was applied to owners or managers of 88 DP units. Specifically, it was applied in all registered and active production units that agreed to participate in the study after an explanation of its objectives. The number of people and animals in each unit was determined, as well as the ownership of cats. The age of cats was segmented into three groups: kittens (3–6 months), juvenile (6–12 months), and adults (> 12 months), as proposed by Györke et al.[18] Sex, the type of food supplied, and the use of sandboxes were also recorded.

Blood sampling and serology

Consent was obtained from the owners of each production unit. All cats included in this study lived in the CAIT, were older than 3 months and no pregnant or lactating female cats were included. Blood samples were taken from the cephalic vein. Then, the serum was obtained and IgG anti-*T. gondii* antibodies were detected by indirect

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ELISA, a test with sensitivity and specificity of 92 % and 88 %, respectively.[15, 19] Serum dilution was 1:200, while cat anti-IgG conjugate dilution was 1:10 000 (SIGMA, USA). Association of seropositivity with risk factors was assessed by odds ratio (OR) with a 95 % confidence interval (CI), using the software Epi INFO v.7.2.2.6 (Centers for Disease Control and Prevention, Atlanta, GA).

Necropsy

Seropositive animals were euthanized.[20] In all cases, consent from the business manager or owner was obtained. Necropsy was performed as described by Aluja and Constantino.[21] Samples from brain, heart, diaphragm, skeletal muscle, lung, spleen, and liver tissues were collected for histopathological study and DNA extraction.

Histopathology and immunohistochemistry

Tissue samples were placed in phosphate-buffered 10 % formalin for at least 24 h in a sample-formalin 1:10 ratio. Once fixed, tissue sections were cut and included for automated processed on histokinette (Leica, Wetzlar, Germany). Then, 5 µm thick sections were cut with a standard microtome (Leica, Wetzlar, Germany), mounted in conventional slides, and stained with the hematoxylin-eosin technique (HE) as previously described, with some modifications.[22, 23]

To confirm morphological identification of *T. gondii*, immunohistochemical assays were carried out with streptavidin biotin-peroxidase complex. The tissues were mounted on electrocharged slides (Biocare), deparaffinized, hydrated, and blocked. Then, the tissues were incubated with primary anti-*T. gondii* polyclonal antiserum (mouse serum positive to *T. gondii* by indirect ELISA), and then with biotinylated secondary antibody (Invitrogen). The reaction was developed with chromogen solution (Betazoid-DAB, Biocare). Sections were contrasted with hematoxylin and covered with hydrophobic resin (Ecomount, Biocare).[23] Slides for histopathologic and immunohistochemistry studies were checked by conventional optical microscopy (Zeiss Axiostar plus; Göttingen, Germany).

DNA extraction

Genomic DNA from brain, heart, and diaphragm was extracted using the Qiagen Gentra® Puregene® Tissue kit (Hilden, Germany), following the manufacturer's instructions. Tissue samples were macerated and incubated overnight in lysis solution and proteinase K (20 mg/mL), subsequently, the DNA was precipitated with isopropanol and hydrated with a commercial solution. The DNA obtained was quantified using a UV-VIS spectroscopy NanoDrop™ 1000 (Thermo Scientific, MA, USA) and kept frozen at -20 °C.

Molecular diagnosis

A TaqMan fluorescent probe-based real-time PCR assay was used to detect the parasite DNA, identifying the specific 62 bp fragment of the *T. gondii B1* gene.[24, 25] Each sample was assayed by triplicate; an exogenous internal positive control (IPC)

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and a negative control were included in each assay. Fluorescent amplification was performed in a StepOne™ thermocycler (Applied Biosystems, Foster City, CA, USA). DNA from the *T. gondii* strain RH was used as a positive control. Samples were also assayed by endpoint PCR, using the *B1* gene and the 529 bp repetitive region[26, 27] as targets.

Statistical analysis

The cats included in this study were selected by a non-probabilistic method, which depended on the acceptance of the owners to carry out the study, the presence of non-owned animals, as well as the consent for euthanasia of those that tested positive. The possible risk factors were analyzed univariately by calculating odds ratios, their confidence interval, and their statistical significance by the chi-square test, using the EPIINFO program v.7.2. If two or more variables were significant ($P \leq 0.1$), a multivariate logistic regression analysis was performed.

Results

In total, 88 DP units were included in this study. Cats were found in 52 of these units. At least one pet cat lived in 29 DP units (32.9 %), and 42 DP units (47.7 %) had stray cats, whereas the presence of rodents was reported in 56 DP units (63.6 %).

Cat data

Ownership. Seventy domestic cats were included in this study. Of these, 47 (67.1 %) felines had an owner, whilst 23 animals were stray. Twelve out of 70 cats (17.1 %) were positive for *T. gondii* antibodies; their location in the production units is shown in Figure 1. Positivity rate was 10.6 % in owned cats, while it was 30.4 % in strays (OR = 3.67, 95 % CI: 1.01–13.27, P = 0.0498) (Table 1).

Sex. From the total population, 43 (61.4 %) animals were female and 27 (38.6 %) were males. With respect to positivity, 8 subjects were females (18.51 %) and 4 were males (14.8 %); no significant differences were observed between sexes (OR = 1.32, CI = 0.32-5.43; P = 0.6821).

Age. Four out of 22 animals aged 6–12 months (18.2 %) were seropositive, while 8 out of 42 cats older than 12 months (19.0 %) were positive. All cats younger than 6 months were negative for anti-*T. gondii* antibodies (P = 0.5052).

Feeding. Information on feeding was available only for the 46 (97.8 %) owned cats. Among them, 12 (26.1 %) were fed raw meat and/or raw viscera, 20 (43.5 %) were fed leftover food, and 14 (30.4 %) were fed commercial/balanced meal only; no information on diet was given for one cat. The highest seropositivity rate was observed in the first group, with 4 positives (33.3 %), while only one positive cat was found in the second group (5 %), and none in the third (P = 0.0131).

Use of sandbox. No owner reported having litter trays for their cats to defecate.

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Figure 1. Location of positive cats for *Toxoplasma gondii* at the CAIT, Hidalgo, Mexico.

Table 1. Characteristics of the cats at CAIT, and seropositive to *T. gondii*

Sex	Age (months)	Ownership		Total
		Owned	Stray	
Males	< 6	2	0	2
	6 - 12	6	3	9
	> 12	10	6	16
	Subtotal	18	9	27
Females	< 6	3	1	4
	6 - 12	10	3	13
	> 12	16	10	26
	Subtotal	29	14	43
	Total cats	**47**	**23**	**70**
	Seropositive to *T. gondii*	5 (10.6 %) *	7 (30.4 %) *	12 (17.1 %)

* Difference between owned and stray cats (OR = 3.67, CI 1.01-13.27, P = 0.0498)
OR = Odds ratio, CI = Confidence interval

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Multivariate analysis

Only two variables were found to be significant: type of cat (owned or non-owned) and diet. However, a logistic regression could not be performed, as information on the second variable was only available for owned animals.

Necropsy and microscopic findings

Post-mortem studies were only performed on 5 of the 12 seropositive cats, which were those authorized by the owners of the properties. Three females and 2 males. No acute or chronic lesions suggestive of *T. gondii* infection were observed, neither macroscopically nor microscopically. By immunohistochemistry, all tissues were negative for *T. gondii*.

Toxoplasma gondii *detection by PCR*

Four out of 5 cats were positive for *T. gondii* DNA in at least one of tissue samples by qPCR and/or end point PCR. With the *B1* hnPCR gene, the parasite was only detected in the brain of one cat; with the 529 bp repeat PCR gene, the parasite was detected in two animals: one in the heart and the other in the diaphragm; finally, with the *B1* gPCR gene, the parasite was detected in four animals: two in the heart, one in the diaphragm and two in the brain. It should be noted that the parasite was isolated from one of the cats by inoculation in mice (Figure 2).

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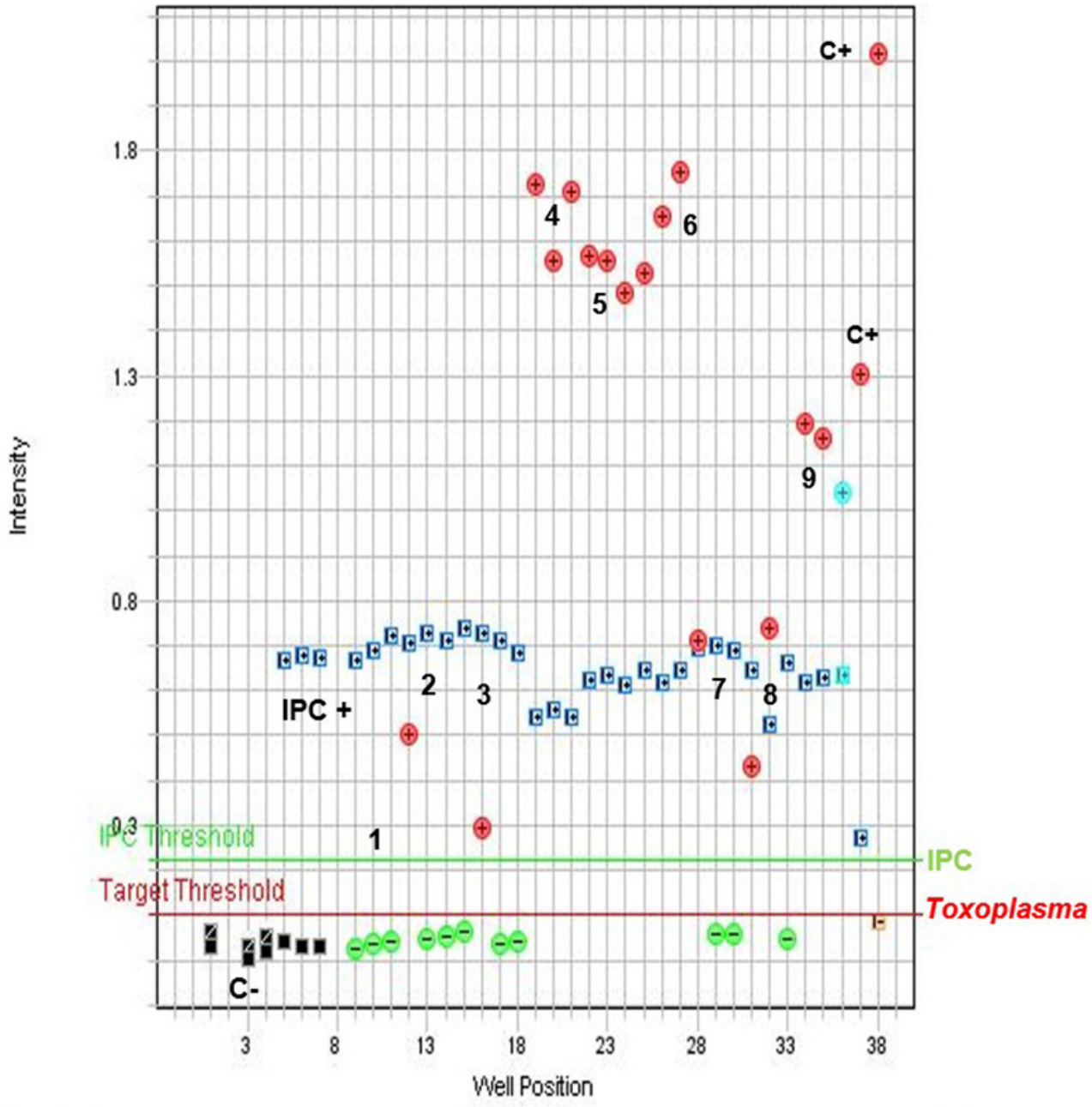

Figure 2. Example of real time PCR detection of the parasite DNA by presence/absence assay. Each PCR reaction contains two sets of primers/TaqMan probe to detect *B1 T. gondii* gene and the IPC (internal positive control). The black squares correspond to C- (control-blocked IPC wells), while the blue squares show IPC+. The green circles correspond to negative amplification for *B1 T. gondii* gene, while red ones show a positive amplification of *B1 T. gondii* gene. 1. Diaphragm (cat 1); 2. Diaphragm (cat 2); 3. Brain (cat 3); 4 Heart (cat 2); 5. Heart (cat 3); 6. Brain (cat 4); 7. Diaphragm (cat 5); 8. Diaphragm (cat 4); 9. Brain (cat 5). C+: DNA of RH strain.

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Discussion

Cats and other feline species are considered as the epidemiological axis of toxoplasmosis, since their direct or indirect interaction with various hosts, in a complex dependence with other factors, conditions the infection and allows the perpetuation of the biological cycle of the parasite.[3, 5–7] The positivity rate found herein (17.1 %) is lower than those reported for other regions in Mexico, which range from 21 to 63 %.[12–15] According to Dubey,[7] *T. gondii* seroprevalence varies in different geographic areas, being higher in warm climates/mild-humid zones than in colder, drier mountainous areas, as the former favor oocyst formation and survival. The climate in Tizayuca, Hidalgo, is dry, with rain only in the summer, and oocyst sporulation may not be favored in the dry season.

The higher seropositivity rate found in stray cats is explained by the fact that these animals live outdoors and have a greater chance to hunt intermediate hosts (birds and small mammals).[18, 24] This is relevant because of the closeness of stray cats with cattle, dogs, and humans.[7] The lack of differences in seropositivity by sex in our study contrasts with findings by other authors, who have reported a higher frequency in females.[14] Regarding the use of litter boxes, the fact that owners do not find it necessary may be because, in rural environments, owners consider that there is enough space for their animals to defecate anywhere outside the house. This point highlights the need to apply hygienic measures that help to reduce the transmission of the parasite by preventing open defecation.

With respect to age, the higher positivity rate found in adult animals is consistent with previous reports that the likelihood of contact with infectious stages of *T. gondii* increases as cats age and continue to hunt.[12, 25] Similarly, the higher positivity rates found in cats that eat raw meat are not surprising; tissues commonly fed to cats, especially parts of the central nervous system as well as skeletal and cardiac muscle, may contain *T. gondii* cysts.[15, 18, 25] Notably, despite seropositivity, no macro- or microscopic *T. gondii* lesions were found. This could be related to the stage of infection in the hosts, their physical condition and age, as the cysts may have degenerated over time.[26] Conversely, it is possible that recently established cysts may not have reached a large enough size to be observable or even detected by immunohistochemistry.

The presence of *T. gondii* in the brain, heart, or diaphragm of 4 of the 5 seropositive animals was demonstrated by molecular assay. Detection of the parasite in these locations is considered indicative of chronic infection, suggesting that the cats were exposed to the parasite weeks or even months prior to euthanasia. This could be correlated with the pathological results, in which no tissue cysts were observed. The fact that a seropositive cat tested negative to the molecular assay in all tissues does not necessarily indicate a false-positive ELISA, as other regions or organs could harbor small tissue cysts that were not sampled. The disparity of results between diagnostic techniques when clinical samples are used may be due to the presence of different stages of infection in the hosts, to their physical and immunological status, and to a random distribution of parasites in the host.[28, 29]

The findings of this study are relevant, since CAIT is a dairy production area, and many animals are destined for human consumption at the end of their productive life. Therefore, the persistence of the *T. gondii* life cycle under these conditions could pose a risk to public health. In this regard, Blaga et al. reported a seroprevalence of 17.38 % in bovine carcasses in a French slaughterhouse.[30]

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This is important, considering that one form of transmission is the consumption of raw or undercooked meat, a growing practice in our environment.

Furthermore, the fact that *T. gondii* circulates in an environment such as the dairy region is relevant, since Manzini et al. reported having found *T. gondii* in 17.65 % of milk tanks in Brazil by molecular techniques.[31] In addition, Dubey et al. reported that toxoplasmosis was the cause of five outbreaks attributed to food poisoning.[32] In addition to the presence of susceptible species, Alvarado-Esquivel et al. noted that environmental factors such as altitude, temperature, and mean annual precipitation of 1 266–1 650 mm influence the frequency of parasitosis.[33] In this regard, Tizayuca has favorable conditions, with a precipitation of more than 2 000 mm annually.

In conclusion, the presence of *T. gondii* in cats in CAIT confirms a potential risk of infection for humans. Thus, the presence of the parasite in livestock, dogs, wild animals, and local inhabitants of the area (intermediate hosts) should be evaluated. Further research is needed on dairy cows and their products and by-products, as well as providing information to owners on the proper handling of cats at these sites. Our results on the frequency of *T. gondii* infection and the low levels of molecular identification suggest a low infectious pressure in the area at the time of the study. In addition, the parasite should be isolated to compare its phenotype with isolates from different species in central Mexico.

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Data availability

The original datasets used in this research and if applicable, supporting information files, are deposited and available for download at the SciELO Dataverse repository.

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Conflicts of interest

The authors have no conflicts of interest to declare.

Author contributions

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