First molecular evidence of *Toxoplasma gondii* in synanthropic rodents (*Mus musculus* and *Rattus rattus*) captured in Yucatan, Mexico

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SUMMARY

Toxoplasma gondii is an obligate intracellular parasite recognized as the etiological agent of Toxoplasmosis, a zoonotic disease endemic in different countries worldwide. In Mexico, the disease is recognized as a human and animal health problem. *T. gondii* sporozoites have the capacity of been released from the gut lumen and infect several tissues, including kidneys. Rodents have been identified as intermediate hosts of *T. gondii*; nevertheless, no previous reports exist in Yucatan, Mexico, about their role in the infectious cycle. A total of 187 specimens (130 *Mus musculus* and 57 *Rattus rattus*) were studied. Total DNA was extracted from renal tissue and was utilized in the identification of *T. gondii* with the end-point Polymerase Chain Reaction (PCR) method. Six positive products were purified and sequenced in order to confirm and validate the results through the BLAST Sequence Analysis Tool. The PCR provided a global positivity of 5.34% (10/187). The BLAST analysis found a direct homology for *T. gondii* with identities and coverage of 96-98% and 98-100%, respectively. Our results confirm the role of *M. musculus* and *R. rattus* in the biological cycle of *T. gondii* and offer knowledge of the parasite transmission in intermediary hosts of the region.

Keywords: *Toxoplasma gondii*, *Rattus rattus*, *Mus musculus*, Kidneys, Yucatan, Mexico

RESUME

Première identification moléculaire de *Toxoplasma gondii* dans des rongeurs synanthropiques (*Mus musculus* et *Rattus rattus*) du Yucatan, Mexique.

Toxoplasma gondii est un parasite intracellulaire obligatoire et l'agent causal de la toxoplasmose, une zoonose endémique dans différents pays. Au Mexique, la maladie est reconnue comme un problème de santé humaine et animale. Les sporozoïtes de *T. gondii* peuvent être libérés de la lumière intestinale et infecter différents tissus, dont les reins. Bien que les rongeurs soient reconnus comme des hôtes intermédiaires de *T. gondii*, il n'existe aucune étude sur leur rôle dans le cycle d'infection dans la région du Yucatan au Mexique. Au total, 187 spécimens (130 *Mus musculus* et 57 *Rattus rattus*) ont été étudiés. L'ADN total a été extrait du tissu rénal et utilisé pour l'identification de *T. gondii* par PCR. Six amplicons ont été purifiés et séquencés afin de confirmer et valider les résultats en utilisant l'outil d'analyse de séquences BLAST. La PCR a donné une positivité globale de 5.34 % (10/187). L'analyse BLAST a donné une homologie directe avec *T. gondii*, avec des identités et couvertures de 96-98% et 98-100%, respectivement. Nos résultats confirment le rôle de *M. musculus* et *R. rattus* dans le cycle biologique de *T. gondii* et apportent de la connaissance quant à la transmission du parasite dans des hôtes intermédiaires de la région.

Mots-clés : *Toxoplasma gondii*, *Rattus rattus*, *Mus musculus,* Reins, Yucatan, Mexique

Introduction

Toxoplasmosis is a parasitic disease distributed worldwide caused by the protozoan *Toxoplasma gondii*, which can infect birds and mammals, including humans. The disease is important in pregnant women and immunosuppressed people. It is estimated that up to 30% of the human world population is suffering chronic infection with generally benign or mild nonspecific clinical symptoms. In Mexico, is considered a public and animal health problem [15].

The most common sources of human infection worldwide are: ingestion of tissue cysts (with bradyzoites) in undercooked meat, or of food or water contaminated with oocysts, and transplacental transmission. The definitive host of *T. gondii* are individuals of the *Felidae* family, including domestic cats (*Felis catus*) [12].

Rodents have an important role as intermediate hosts of *T. gondii*, and are involved in the infection cycles domestic, peridomestic and wild [24]. Furthermore, rodents can be use as sentinels in the way that *T. gondii* spreads in urban and rural areas, as they are exposed without any protection to all the infective forms of the parasite and feed on various sources of food on the contaminated ground [18].

Predation of these animals is a fundamental element for the dissemination of *T. gondii*, because they are important sources of infection for domestic and wild cats [9]. Rodents represent two thirds of the diet of medium cats and other wild life species, although this varies by factors such as population density and the presence of other potential prey [2].

In Yucatan, Mexico, there are reports of *T. gondii* circulation in humans [30], domestic animals [4, 20], and sylvatic animals [29]; in these studies, are described high rates of seroconversion or molecular frequency, demonstrating the wide distribution of *T. gondii* in the region. However, the active involvement of synanthropic rodents in the infectious cycle of toxoplasmosis has not been previously documented in Yucatan.

The aim of this study was to use a conventional PCR to investigate the presence of *T. gondii* in biological samples from synanthropic rodents (*Mus musculus* and *Rattus rattus*) captured in a rural community from Yucatan, Mexico. The molecular diagnosis tests in Toxoplasmosis are amply used in animal and human medicine principally for their higher sensibility in the detection of positive cases. Also, are the best option if the serological tests are not available [5].

Materials and methods

STUDY AREA

The study was conducted in the rural community of Molas, Yucatan, Mexico (20°40'N, 89°38'W), located within the ecological reserve "Cuxtal". The climate in the region is warm subtropical with summer rains (Aw 0), with an average annual temperature of 26°C. The predominant vegetation is deciduous forest and the land use is mainly for farming, and secondarily for urban settlements [16].

SELECTION OF HOUSINGS, RODENT CAPTURE AND SAMPLING

For selection of housings, the total area of urban settlement in the study site was divided into four quadrants by drawing two imaginary axes crossing through its center. In each quadrant ten houses were selected for convenience; in each house a total of 12 Sherman traps (7.5 x 23 x 9 cm, HB Sherman Traps Inc®, Tallahassee, Florida, United States) were placed inside and outside of the construction. The quadrants were sampled one after another, one week per month, during all the period sampling: October and December 2011 (rainy season) and January to March 2012 (dry season). The capture effort was 4,320 traps/night per period. The traps were baited with a mixture of oats and artificial vanilla flavoring, and placed in the morning and revised the following day.

The trapping was conducted with permission of the *Secretaría de Medio Ambiente y Recursos Naturales* (SEMARNAT) of Mexico (Registro SGPA/DGVS/02528/13) and under the statutes of the American Society of Mammalogists (ASM). All rodents captured were transported alive to the Zoology Laboratory of the *Campus de Ciencias Biológicas y Agropecuarias* (CCBA) of the *Universidad Autónoma de Yucatán* (UADY), where conventional somatic measurements were taken, and age, species and sex were determined. After, were anesthetized with ether, and then sacrificed by cervical dislocation. The necropsy was conducted for the collection of biological samples in the internal organs.

DNA EXTRACTION

Total DNA was obtained from the renal tissue using the Trizol extraction method (Ambion®, Life Technologies, California, United States) following the manufacturer's specifications. We were in the necessity to use kidneys because these were the only biological material available for the present study; brain and others tissues have been employed in different investigations.

A whole kidney was macerated using a mortar and pestle that had been previously sterilized in an autoclave and UV light, in order to avoid cross contamination. The total extracted DNA was stored at -80°C until use in the molecular test. The concentration and purity of nucleic acids were determined by means of a spectrophotometer (NanoDrop 2000™, Thermo Scientific ®, Wilmington, United States).

POLYMERASE CHAIN REACTION (PCR)

The end-point PCR was performed using the primers used by Boughattas *et al.* [1]: B22: AACGGGCGAGTAGCACCTGAGGAGA (sense) and B23: TGGGTCTACGTCGATGGCATGACAAC (antisense), which amplify a fragment of 115 bp immerse in the gene B1 of genome of *T. gondii*. The reagents used in the reaction had the following final concentrations: PCR Buffer 1X, 2.5mM $MgCl^2$, 0.2mM dNTP´s, 0.2mM of each primer, 1U Taq polymerase (Thermo Scientific®, Inc., Waltham, Massachusetts, United States) and distilled water. The thermocycler conditions were: initial denaturation at 95°C for five minutes, followed by 35 cycles at 95°C for one minute, 58°C for 45 seconds and 72°C for 45 seconds; the final extension was 72°C for 10 minutes. All reactions included positive (total DNA from a brain of a rodent [BALB/c] experimentally inoculated with *T. gondii*) and negative controls (sterile water). Electrophoresis was performed in polyacrylamide gels 8%, stained with ethidium bromide.

PURIFICATION, SEQUENCING AND ANALYSIS

For purification of PCR positive amplicons, a new electrophoresis was realized in agarose gels 1%, stained with ethidium bromide. The 115 bp band, was removed using a scalpel, and purified with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research®, The Epigenetics Company™, United States) according to the manufacturer's specifications. Six purified products were sent to sequence to the *Instituto de Biotecnología* (IBT) of the *Universidad Nacional Autónoma de México* (UNAM). The sequences obtained were compared with other sequences of the GenBank® using the Basic Local Alignment Search Tool® (BLAST), by the National Institute Health (NIH, United States).

STATISTICAL ANALYSIS

The variables of the captured animals (species, sex and age), and the PCR results were analyzed with descriptive statistics. Additionally, a χ^2 test was used to establish the association (IC 95%, $P<0.05$) between the independent

variables (species, sex and age) and the results of the PCR (dependent variable). Data were analyzed with the program PASW™ STATISTICS 18 (SPSS Inc. 233, Chicago, IL).

Results

A total of 187 synanthropic rodents were captured: 69.5% (130/187) belonged to the *M. musculus* species, and 30.5% (57/187) to the *R. rattus* species. All them apparently healthy under the external physical examination. The rodent species were identified according to the key of Reid [23].

The PCR test provided a positivity rate per specie of 6.92% (9/130) for *M. musculus*, and 1.75% (1/57) for *R. rattus*. The overall positivity was 5.34% (10/187). In Figure 1, is shown the representative gel with positive PCR fragments.

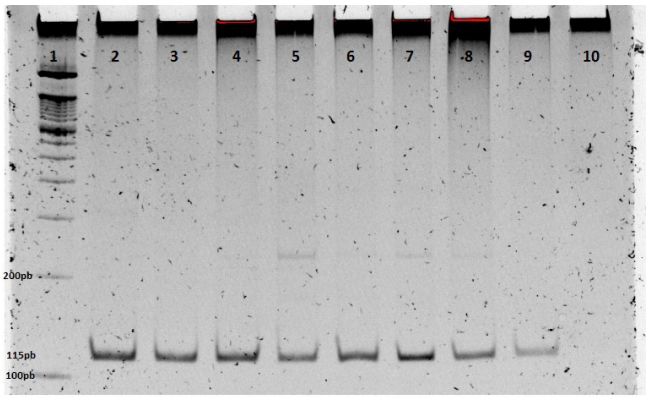

Figure 1: Polyacrylamide gel 8%, stained with ethidium bromine, showing the positive extractions (1: Molecular weight ladder (100 bp); 2: Positive control; 3-9: Positive samples; 10: Negative control).

In the Table 1, are shown for each one of the products, sequences, and identity and coverage percentages, obtained in the BLAST analysis. All sequences were homologous to the *T. gondii* VEG strain with accession number LN714499.1. Unfortunately, due to the size of our amplified PCR fragments (less than 200 bp), it was not possible to obtain an access number in the GenBank® (this fact was not known by any of the authors involved in this investigation).

We did not find a statistically significant association between the independent variables and the dependent variable ($P>0.05$), probably due to the low number of positive individuals.

Discussion

Synanthropic rodents are widely distributed in Mexico. Particularly, the species *M. musculus* and *R. rattus* are the most abundant in peridomestic and domestic environments in Yucatan, Mexico, due to different factors as food sources, climate and poor hygiene conditions [21]. These species have been identified in Yucatan, as carriers or reservoirs of several and important zoonotic agents such as *Leptospira* spp. [28], *Rickettsia felis* [22], *Trypanosoma cruzi* [33] and different Flavivirus [6].

In our PCR test, we decided to use the primers B22 and B23, because, in molecular tests conducted in tissues, they were the most sensitive and specific ones [5]; however, any negative result in this investigation, has to be interpreted carefully because it is possible that *T. gondii* could be present in the unexamined tissues or organs of the used rodents.

According to the results obtained by our PCR and the subsequent sequence BLAST analysis (Table I), *T. gondii* DNA is present in the kidney tissue of *M. musculus* and *R. rattus* captured in Yucatan. This converts to both studied species in potential transmission pathways, which represents an epidemiological risk to domestic animals and inhabitants of community studied [28].

Even though the kidney is not an organ referenced in the Toxoplasmosis investigation, as such as brain and hearth; in *T. gondii* infection, sporozoites are released from oocysts in the gut lumen of the host, subsequently infects the intestinal

Products	Sequence	Identity %	Coverage %
Mm 89	AGAGTACTAGAACAGTCGCCGCTACTGCCC AGTTGTCATGCCATCGACGTAGACCCA	96	100
Mm 230	TAGAGAGTACTAGAACGTCGCCGCTACTGCC CAGTTGTCATGCCATCGACGTAGACCCA	98	100
Mm 304	CATAGAGAGTACTAGAACGTCGCCGCTACT GCCCAGTTGTCATGCCATCGACGTAGACCCA	98	98
Mm 374	TAGAGAGTACTAGAACGTCGCCGCTACTGCC CAGTTGTCATGCCATCGACGTAGACCCA	98	100
Mm 378	TAGAGAGTACTAGAACGTCGCCGCTACTGCC CAGTTGTCATGCCATCGACGTAGACCCAA	98	98
Rr 214	TAGAGAGTACTAGAACGTCGCCGCTACTGCC CAGTTGTCATGCCATCGACGTAGACCCA	98	100

Mm: *Mus muculus*
Rr: *Rattus rattus*

Table I: Name and sequence of positive PCR products and the percent identity and coverage obtained by BLAST analysis. The percentage products were obtained in comparison with *T. gondii* VEG strain with accession number: LN14499.1.

epithelium and through the blood stream, invades several tissues (brain, heart, eye, kidney, liver, spleen and lung), causing different signs and symptoms depending on the site of infection [8]. Additionally, Dubey and Frenkel [10], mentioned that parasites in rodents with proven infection, do not always encyst in the brain. It seems that the host immune system induces mutations in the *T. gondii* genes that control the parasite's ability to form cysts, as well as its pathogenicity. Furthermore, many studies made in different species, have demonstrated natural infection in kidneys: *T. gondii*-like tachyzoites, were found associated with necrosis in intestine, spleen, liver and kidneys, from a Blanford's fox (*Vulpes cana*) [11]; also, a mixed breed female dog from Brazil, had positive PCR results in tissue kidney [27], and, in a Valley quail (*Callipepla californica*) were found large numbers of *T. gondii* tachyzoites in kidneys [3]. Relevant is the study of Epiphanio *et al.* [14], in which were found several species of new monkey world, positives to immunohistochemistry to *T. gondii* infection in kidney tissue.

The information generated in Mexico about the role of synanthropic rodents in the infection cycle of *T. gondii* is limited and scarce. There is a study conducted in the Durango State by Dubey *et al.* [12], with a seroprevalence report of 0.8% to 3.1% for mice and rats, respectively. Also, Roch and Varela [26], reported circulating antibodies against *T. gondii* in 350 (36.3%) rats captured in Mexico City. For this reason, we believe that the present study, is maybe, the first molecular evidence of *T. gondii* natural infection in synanthropic rodents from Mexico; which can be corroborated in the published revision of Hernández-Cortazar [15]. Rendón-Franco *et al.* [25], made previously a molecular evidence of *T. gondii* natural infection, but their study was conducted in wild rodents.

Worldwide, several recent studies using molecular essays have shown significant variations in the detection frequency of *T. gondii*. For example, Yan *et al.* [32], reported a frequency of 22.3% in *M. musculus* individuals, captured in the urban area of Xuzhou, China. In this same species, Vujanić *et al.* [31], reported a frequency of 10.4% in individuals captured in three locations from Belgrade City (Serbia), characterized by poor housing and degraded environment. Similarly, Elamin [13], in a study conducted with the *R. rattus* species, found a molecular frequency of infection of 11%. These studies show a higher prevalence compared to the obtained in our molecular test. Likewise, Kijlstra *et al.* [17], found a prevalence of 6.5% in *M. musculus* individuals, captured in pig farms in the Netherlands; a percentage very similar to established in our study. In contrast, in a study conducted with urban rodents captured in São Paulo, Brazil, Muradian *et al.* [19], reported the presence of *T. gondii* DNA in two of 121 animals, which results in a frequency of 1.65%, a result lower than ours. All these studies are evidence of the great acceptation of the molecular assays for the detection of *T. gondii* infection in animals, because tend to show a higher positivity rates in comparison with serological assays [27].

In this study it was not possible prove the association between the distribution of *T. gondii* with the characteristics of the study rodents; however, *T. gondii* infection in rodents probably involves the interaction of complex ecological elements. The difference between infection rates may be due to environmental factors, especially variations in temperature and relative humidity that allows the survival of *T. gondii* [15]. Likewise, *T. gondii* prevalence in rodents is influenced for by the food and shelter availability and therefore, the abundance and distribution of rodents and their predators [7, 18, 24].

Is widely recommended, conduct future epidemiological studies in the region, using analysis of multiple *T. gondii* markers to identify circulating genotypes in *R. rattus* and *M. musculus* [12]. Similarly, other species of intermediate hosts should be included in order to identify the distribution of *T. gondii* in wild and peridomestic environments and expand epidemiological knowledge of Toxoplasmosis [15, 31].

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