Toxoplasma gondii infection in European mouflons (Ovis musimon) and captive wild felines from Puebla, México

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ABSTRACT

The presence of Toxoplasma gondii in zoo is cause of alert because many susceptible species kept in captivity die of clinical toxoplasmosis. Moreover, excretion of T. gondii oocysts by infected captive wild felines into the facilities could pose a risk to workers. Herbivores in wild collections can serve as sentinels of local transmission, since they get infected by the consumption of oocysts present in ground or water. Both herbivores and felids may reveal the parasite variants which are circulating in the region. We determined the seroprevalence of T. gondii in European mouflons (n = 55) and wild felines (n = 15) from a private zoological collection located in the Eastern region of México, as well as the incidence in 41 of the mouflons using ELISA. The prevalence of T. gondii in mouflons was 14.5% (n = 55) and 17.1% (n = 41) in 2011 and 19.5% in 2012. The estimated incidence was 9.8%-12.2%. In wild felines the frequency was 80%. Four sero-positive animals (two mouflons and the two oldest African lions) were euthanized. Histopathology, conventional PCR (for BI and SeqRep529 loci) and molecular characterization were carried out. All euthanized animals were positive to T. gondii by PCR. We identified a triple infection (I + II + III) in the brain of a mouflon. In conclusion, a high infective pressure of T. gondii in the collection was found, supported by changes in its prevalence in European mouflons. A high prevalence of infection in wild felines was determined. At least four genotypes of T. gondii are present in herbivores and carnivores, and one mouflon had a mixed infection.

1. Introduction

Toxoplasma gondii is a cosmopolitan pathogen that infects almost any warm-blooded vertebrate, i.e., mammals and birds. The success of the parasite lies in its ability to reproduce both sexually and asexually among different hosts (Grigg and Sundar, 2009). Susceptible species kept in captivity such as Australian marsupials, New World monkeys, lemurs and meerkats, can die due to toxoplasmosis (de Camps et al., 2008). If a feline kept in captivity is infected, it represents an added risk to infect staff (de Camps et al., 2008; Alvarado-Esquivel et al., 2013). Ungulates in semi-confinement wild collections can serve as sentinels of environmental contamination; for instance, herbivores can become infected by the consumption of oocysts while grazing or drinking water. Besides this, carnivores can be infected by the ingestion of raw meat containing T. gondii tissue cysts (Ferreira et al., 2019). There are few studies of T. gondii infection in private or public collections of wild animals in México (Espinosa-Avilés and Martínez-Morales, 2007; Alvarado-Esquivel et al., 2013), and none in the Eastern region of the Country, which has favorable climatic conditions for T. gondii and is adjacent to one of the most populated urban regions of México. The studied animals are in semi-confinement, which may offer a greater exposure to the parasite than traditional zoos. The present study was aimed to determine seroprevalence of T. gondii in European mouflons (Ovis musimon) and wild felds (Panthera leo and P. tigris) that live in semi-confinement in a private zoological collection. We also detected DNA of T. gondii in tissues samples of animals as well as determined genotypes present there. This

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is a cross-sectional prospective study and not from serum banks, as it has been commonly reported. In addition, we genotyped clinical samples collected specifically for this purpose.

2. Material and methods

2.1. Ethical approval

The present study was approved by the reviewing board of the Instituto Nacional de Pediatría of the Ministry of Health of Mexico (INP; IRB-NIH numbers IRB00008064 and IRB00008065), which includes the Research and Animal Care Committees with registration number 013/2012.

2.2. Study site

The study was conducted in a private zoological collection localized at the municipality of Puebla, Puebla (18° 56′ 13.0″N, 98° 08′ 11.0″W), with an altitude of 2117 m above sea level, an annual temperature between 12 °C and 18 °C and annual rainfall from 700 to 1500 mm INEGI, (2020).

2.3. Collection of blood samples

In November 2011, 5 mL blood was collected from the jugular and saphenous veins from 55 randomly selected adult European mouflons (1–5 years old) and 15 wild felids [eight African lions (Panthera leo), and seven Bengal tigers (P. tigris)], respectively. The proportion of individuals in the population that had anti-\textit{T. gondii} antibodies at the time of sampling (prevalence) was determined. In November 2012, we found 41 of the 55 mouflons; they were sampled again and the incidence of \textit{T. gondii} infection was determined (number of new cases of a disease in one year).

2.4. Serology

To detect specific antibodies against \textit{T. gondii} in European mouflons and wild felids, we standardized an indirect ELISA using crude extract of the RH strain as antigen was performed as per Olamendi-Portugal et al. (2012), with some modifications; sera of mouflons and wild felids were diluted 1:400 and 1:200, respectively. Due to unavailability of a specific conjugate against mouflons, some secondary antibodies (anti-sheep, anti-goat, anti-bovine and protein G) were tested by direct ELISA using mouflon sera. The anti-goat conjugate was chosen because it recognized mouflon IgG with the highest sensitivity. Mouflon sera were tested with 1:10,000 dilution of the rabbit anti-goat IgG peroxidase conjugate (Sigma–Aldrich Corp., St Louis, MO, USA) (Supplementary figure). For sera of wild felids, the secondary antibody against cats coupled to peroxidase (Abcam, Cambridge, MA, USA, product ab112801) was used and diluted 1:15,000 (Valenzuela-Moreno, 2014). For mouflons and felines, three negative and three positive serum samples of naturally infected domestic goats and cats, respectively, were used as controls in each plate to determine the cut-off value, which was the mean plus 3 standard deviations of the values obtained with the three negative controls tested in duplicate, i.e., from six wells (Caballero-Ortega et al., 2008; Valenzuela-Moreno, 2014). Taking the absorbance of the positive
goat sera (direct positive control) as 100 percent, the absorbance obtained in each well (direct and experimental mouflon samples) was corrected. The modified absorbance value of each mouflon serum was divided by the cut-off point to establish the Reactivity Index (RI). The RI frequency and the data correlation between 2011 and 2012 samples were plotted using frequency distribution curves and a scatter plot (Fig. 1).

### 2.5. Collection of tissue samples

Two mouflons with the highest RI of anti-*T. gondii* antibodies (M1 and M2) as well as the two aged (>24 years old) African lions (L1 and L2) included in the survey were euthanized with Sodium Pentobarbital (133 mg/kg body weight IV Dolasetal®), according to the Mexican official standard NOM-033-ZOO-1995. Lions were previously anesthetized with a mixture of 2.0 mg/kg body weight Xylazine (Procin equus®) plus 4.0 mg/kg body weight Ketamine (Ketamin-Pet®, Aranda) intramuscularly.

The necropsies were performed as per Schunemann and Constantino (2002). Tissue samples from heart, liver, spleen, brain and striated muscle (diaphragm and right gracilis) were collected. After the necropsy, one section of those tissues was fixed in 10% buffered formalin for histopathology, and other was frozen at -20 °C for DNA extraction, molecular detection and genotyping of the parasite.

### 2.6. Molecular assays

Genomic DNA from heart, liver, spleen, muscle and brain was obtained, using Qiagen Gentra® Puregene® Tissue kit (Hilden, Germany), following the manufacturer instructions. For molecular diagnosis, DNA was amplified using previously described primers targeting B1 gene and the non-coding S29 bp repetitive sequence (SeqRep529) (Pujol-Riquel, 1999; Homan and Vercammen, 2000). Multilocus nested PCR assays were performed to amplify 10 molecular markers (SAG1, Alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) following the methodology previously described; the amplified products were digested with specific enzymes (Su et al., 2010). The RH strain DNA and sterile water were included as positive and negative controls, respectively; in addition, positive and negative reamplification controls were also included in nested assays. For genotyping, DNA from RH, Me49 and VEG reference strains (type I, II and III respectively) were included. All PCR assays were carried out with AmpliTag Gold™ DNA polymerase (Thermo Fisher Scientific, cat. 4311806, MA, USA). In order to determine the parasite load in tissues, we followed the methodology previously described (Cedillo-Pelaez et al., 2011), with modifications using primers and TaqMan® probe for SeqRep529 of *T. gondii* designed by Robert-Gangneux et al. (2010).

### 2.7. Histopathology and immunohistochemistry

Representative fixed tissue samples were stained with hematoxylin and eosin. For immunohistochemistry (IHC), a 1:250 dilution of *T. gondii* positive goat serum (previously tested by ELISA and Western blot) was used as the primary antibody. A biotinylated mouse anti-goat IgG antibody (Biocare Medical, CAL, USA) diluted 1:300 was used as a secondary antibody. Immunocomplexes were detected with a commercial solution (Betazoid DAB, Biocare, Chromogen 3, 3' Diaminobenzidine). Sections of liver or spleen of mice infected with tachyzoites of the Me49 strain were used as positive controls; as negative control, sections of the same tissue were used replacing the primary antibody with PBS (Cedillo-Pelaez and MéxicoMéxico, 2015). Both HP and IHC slides were examined by optical microscopy (Zeiss Axiosstar plus; Göttingen, Germany).

### 2.8. Statistical analyses

All data were analyzed using the SPSS v23.0 software (SPSS Inc., Chicago, IL, USA). The correlation between the sampling of 2011 and 2012, as well as between results of the first versus the second assays, was determined by R Pearson and Spearman correlation methods, with 95% confidence limits. Confirmation of the normal distribution of the lower absorbance values population was determined by Kolmogorov-Smirnov (K-S) test. In all cases a p ≤ 0.05 was considered as statistically significant.

### 3. Results

In 2011, two populations of mouflons were identified in the frequency distribution of ELISA results: one grouped to the left of the histogram with normal distribution (K–S p = 0.124), while 8/55 (14.5%) were grouped to the right (Fig. 1A). One year later, 7/41 (17.1%) and 8/41 (19.5%) mouflons sampled in 2011 and 2012, respectively, were positive (Fig. 1B). From the 41 original mouflons re-tested one year later, three remained positive, five became negative and four sero-converted positive; one mouflon was on the cut-off (Fig. 1C, R = 0.35; p < 0.01). Therefore, the annual apparent incidence of *T. gondii* ranged between 9.8% (4/41) and 12.2% (5/41; considering one mouflon on cut-off as positive. Fig. 1C). Also, six out of eight lions (75%) and six out of seven tigers (85%) of the 15 wild felines analyzed were positive for anti-*T. gondii* antibodies, resulting in an overall prevalence of 80% (Table 1).

The two mouflons selected for euthanasia had good body condition (BCS 1.5/5) and had chronic lesions associated with advanced age (AZA Nutrition Advisory Group, 2016; 2017). None of these animals had lesions compatible with those reported for acute or chronic infection of *T. gondii* during the necropsy or histopathology analyses. By IHC, immunopositive structures compatible with cysts or pseudocysts-like of *T. gondii* in the spleen and the muscle of mouflon 1 (M1) and brain of lion 2 (L2) were identified (Fig. 1D). We did not find *T. gondii* tissue cysts in the other two animals.

All euthanized animals (n = 4) were positive by conventional PCR of B1 or SeqRep529 markers; in order to increase the chances to get enough DNA for genotyping, we chose those tissues that were positive for both loci to perform the multiplex nested PCR. A mixed infection was identified in the brain of M1 (I + II + III) at SAG3 gene (Fig. 2; Table 2). The markers that could be genotyped were SAG1, Alt. SAG2, SAG3 and Apico, which presented type I and III alleles. BTUB, c22-8 and PK1

### Table 1

| Year | Species       | Scientific name | Sex ratio (male: female) | No. positive/No. tested | Prevalence (%) | Incidence |
|------|---------------|-----------------|--------------------------|------------------------|----------------|-----------|
| 2011 | African lion  | Panthera leo    | 4 : 4                    | 6/8                    | 75.0           | –         |
|      | Bengali tiger | Panthera tigris | 4 : 3                    | 6/7                    | 85.7           | –         |
|      | European mouflon | Ovis musimon | 16 : 39                  | 8/55                   | 14.5           | –         |
|      |                |                 | 10 : 31                  | 7/41                   | 17.1           | <9.8% (4/41) and 12.2% (5/41) |
| 2012 |                |                 |                          | 8/41                   | 19.5           |           |

*One mouflon was on the cut-off point.*
markers amplified the expected product, but due to the low amount of DNA they could not be genotyped. Parasite load could only be calculated in the samples of the mouflons, and values < 50 parasites per mg of tissue were obtained (Table 2).

4. Discussion

Prevention and control of toxoplasmosis is a significant issue in zoos and private collections, as wild felines and feral cats can excrete oocysts in their feces. These parasite forms could be disseminated through the zoo’s facilities by rainfall water and mechanically by the zoo staff; by these means, captive species (some of them listed as endangered) may get infected and die due to toxoplasmosis (de Camps et al., 2008). In the present study we determined the exposure to T. gondii of herbivores and carnivores from a private zoological collection in the state of Puebla, Mexico. The mouflons included in this study live in semi-confinement and therefore could have the possibility of being exposed to oocysts shed by feral and wild felines to water or grass that grows in the meadow. Previous studies showed higher prevalence of T. gondii in wild mouflons (15.0–23.0%) than in mouflons raised in total confinement (0.0–8.0%). We found high seroprevalence of T. gondii (14.5% or 17.1% in 2011 and 19.5% in 2012) as compared to that found in animals (0.0–8.0%) raised in traditional zoos (Hejlícek et al., 1997; Sedlák and Bártová, 2006; Aubert et al., 2010).

As far as we know, this is the first report of incidence of T. gondii in wild sheep (9.8%–12.2%), which was higher than that reported by Caballero-Ortega et al. (2008) in domestic ovises (2.1%) of an experimental farm in a tropical region between Puebla and Veracruz states of Mexico. It must be highlighted that a few months later there was an outbreak of toxoplasmosis in this private collection, which caused the death of some susceptible individuals in the collection (unpublished data). This confirms that herbivores, by feeding on grass, are useful as sentinels of infection by T. gondii oocysts.

High seroprevalence reported in our study is concordant with other studies (Philippa et al., 2004; Yang et al., 2017; Ferreira et al., 2019). For instance, 81.3% of captive wild cats tested in three zoos of Mexico City were seropositive (Alvarado-Esquível et al., 2013). The routine diet of the wild felids included chicken and horse meat, which may contain tissue cysts of T. gondii; previously, cysts were detected in chicken and horse from elsewhere in Mexico (Dubey et al., 2004; Alvarado-Esquível et al., 2012, 2015). Therefore, feeding wild animals in captivity with raw meat may represent a high risk factor of infection by T. gondii (Alvarado-Esquível et al., 2013). Freezing meat at −12 °C for at least 7 days before feeding it to animals is considered to be the most practical strategy to reduce T. gondii infection in carnivores (Dubey, 2010).

Almost all felines were positive, while most mouflons were negative; these results seem to be interesting. It is important to note that all wild felines are old adults (some of them older than 20 years) and throughout their lives they have been constantly exposed to tissue cysts or oocysts of the parasite, unlike mouflons, which are mainly young adults (under 5 years) and are only exposed to the risk of consuming T. gondii oocysts. Alternatively, seronegative mouflons could be infected with T. gondii, but their antibody titers could be below the ELISA detection limit; this may be due to fluctuations in immunoglobulin levels and they have not been re-infected with the parasite or the parasites cyst in the tissues of the mouflons have not been reactivated (Rougier et al., 2017). This is supported by the fact that some mouflons when retested a year later became seronegative while others remained seropositive (green and blue dots in Fig. 1C, respectively).

By conventional anatomicopathological techniques, no lesions associated to acute or chronic infection of T. gondii were identified in sampled animals, which was congruent with lack of clinical manifestations related to T. gondii infection in any of the positive animals. Some immuno-positive small structures were found in the spleen and the muscle of one mouflon and in the brain of one lion by IHC (L2). In clinically healthy animals, it is difficult to find lesions associated with T. gondii infection, thus more sensitive tests, such as IHC and PCR, are required to demonstrate the presence of this infectious agent (Silva et al., 2013).

Surface antigens SAG1, Alt. SAG2 and SAG3 were the genes with the highest amount of positive and genotyped results. This correlates with results previously obtained by our group using clinical samples of humans (mother/newborn pairs), feral cats and stray dogs (Rico-Torres et al., 2018; Valenzuela-Moreno et al., 2019, 2020). Our interest was to genotype T. gondii from clinical samples; therefore, the success in the genetic characterization of this parasite was limited but with interesting results, since we were able to identify a triple infection in the brain of mouflon 1: I + II + III at SAG3 gene. The detection of mixed infections is of particular interest in epidemiological studies, since they demonstrate the circulation of several strains in a specific region that can result in the recombination and generation of new variants when they are simultaneously ingested by felines. In Mexico, the presence of more than one strain in a given host has become rather common, because it has been described in animals from neo-tropical regions of Mexico, i.e. in feral cats from Quintana Roo, stray dogs from Chiapas and pigs from Yucatán (Cubas-Atienza et al., 2018; Valenzuela-Moreno et al., 2019, 2020). However, this phenomenon is not exclusive to those regions, since in human congenital cases from the Valley of Mexico, and now in mouflons from Puebla (both neo-artic regions) we have found mixed infections as well (Rico-Torres et al., 2018).

In conclusion, we found a high infective pressure of T. gondii in the collection, supported by changes in its prevalence in European
Table 2

| ID   | Tissue | SAG1 | SAG2 | b1 gene hmpCR | b29 bp repeat PCR | Tachyzoites/mg tissue | Genetic markers |
|------|--------|------|------|---------------|-------------------|-----------------------|-----------------|
| Mouflon 1 | Brain | I | I | (+) | (+) | 50 | 1, II, III |
| | Liver | (--) | (--) | (--) | (--) | nd | I, II or III |
| Lion 1 | Spleen | (--) | (--) | (--) | (--) | (+) | (+) |
| | Liver | (--) | (--) | (--) | (--) | nd | III |

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mouflons; also, and as expected, the prevalence of infection by *T. gondii* in felines was high. It was also supported by the finding of at least four parasites variants among herbivores and carnivores, as well as a triple infection in one mouflon.

**Declaration of competing interest**

None.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.07.005.

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