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# Prevalence and Genotype Identification of *Toxoplasma gondii* in Wild Animals from Southwestern Spain

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ABSTRACT: We used PCR to detect *Toxoplasma gondii* in the principal game species in southwestern Spain. We detected *T. gondii* in 32.2% of animals tested. Prevalences varied from 14.7% in wild boar (*Sus scrofa*) to 51.2% in red fox (*Vulpes vulpes*). The most prevalent genotype was type II (50.0%), followed by type III (20.6%) and type I (5.9%). Mixed infections (11.8%) were detected in wild boar (types I+III) and red fox (types II+III). Polymorphic strains (11.8%) were detected in several species. The high prevalence and the genetic variability shown could have implications for infection of farm animals and humans.

*Key words:* Genotypes, PCR, PCR-RFLP, Spain, *Toxoplasma gondii*, wild animals.

*Toxoplasma gondii* is an important opportunistic zoonotic pathogen that can infect all warm-blooded vertebrates, causing severe disease in immunocompromised humans and in cases of congenital transmission. Felids are the definitive hosts; other mammals including wild game species act as intermediate hosts that can become infected by ingesting oocysts or parasitized tissues.

*Toxoplasma gondii* has an unusual clonal population structure based on initial analysis of isolates from North America and Europe (Howe et al. 1997). Traditionally, three main clonal lineages or genotypes are recognized: types I, II, and III. Several authors have confirmed the existence of much greater genetic variation in certain geographic locations (e.g., Brazil) and hosts (e.g., wildlife from North America) with the detection of recombinant forms (Ferreira et al. 2006) and atypical strains (Dubey et al. 2011). Subsequently, new genetic types and lineages have been proposed (Pena et al. 2008; Khan et al. 2011).

In Europe, approximately 55.8% of all hunted animals destined for human consumption, commonly in the form of cured meat, contain *T. gondii* cysts (EFSA 2007). Few researchers have genotyped *T. gondii* strains infecting wild or domestic animals in Mediterranean Europe (Richomme et al. 2009; Verin et al. 2013); none in Spanish wild animals. Knowledge of the distribution of genotypes is important because different genotypes may have different degrees of virulence (Pena et al. 2008). Such information would also help define reservoirs, routes of infection, and epidemiologic cycles. We report direct detection and genotyping of *T. gondii* without prior isolation of the parasite from tissue samples of primary game species in southern Spain.

Sampling areas (Fig. 1) were privately owned and public unfenced estates occupying large expanses of dehesa (tree-dotted open pasture) ecosystems in the Extremadura Region (southwestern Spain; 37–41°N, 3–7°W), where game animals share land and natural resources with free-ranging livestock.

Samples of brain or myocardium ($n=183$) were collected from 61 adult wild boars (*Sus scrofa*), 22 red deer (*Cervus elaphus*), 21 fallow deer (*Dama dama*), 12 mouflons (*Ovis aries musimon*), 26 Spanish ibexes (*Capra pyrenaica victoriae*), and 41 red foxes (*Vulpes vulpes*) killed during the 2009–11 hunting seasons.

Ten grams of tissue sample from each animal were digested with trypsin for 1 h, and DNA was extracted from 200-µL aliquots of the digest (QIAamp Mini Kit, Qiagen, Cortaboeuf, France). These were subsequently screened for *T. gondii* using

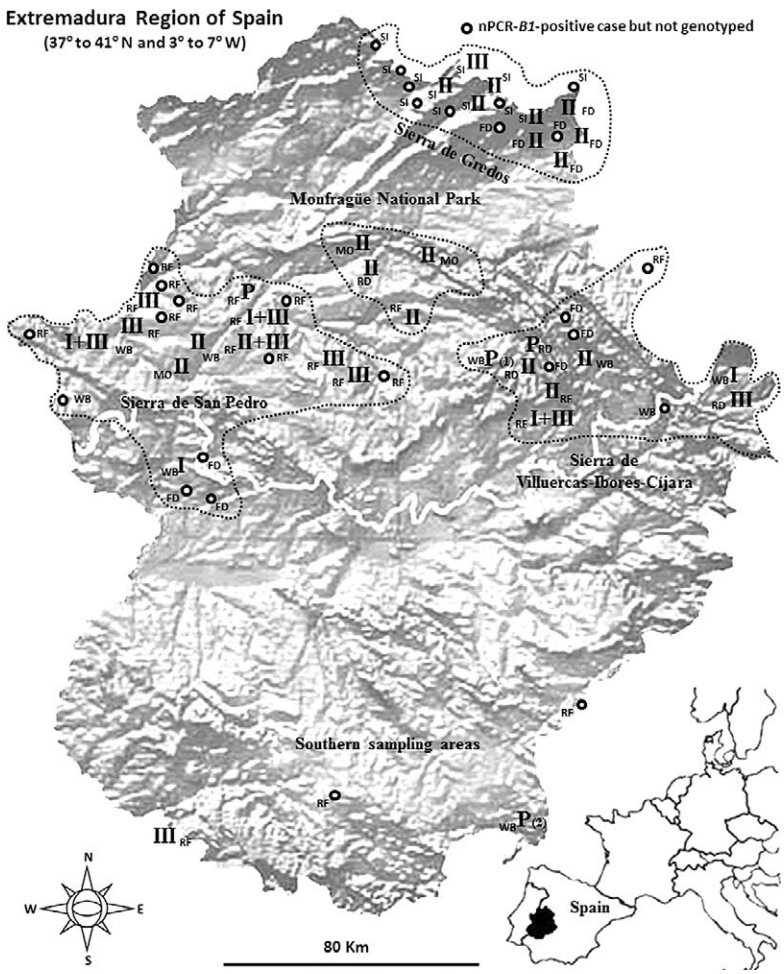


FIGURE 1. Spatial distribution of nested PCR-B1-positive cases and genotypes of *Toxoplasma gondii* detected in wild animal species in southwestern Spain. WB=wild boar (*Sus scrofa*); RD=red deer (*Cervus elaphus*); FD=fallow deer (*Dama dama*); MO=mouflon (*Ovies aries musimon*); SI=Spanish ibex (*Capra pyrenaica victoriae*); RF=red fox (*Vulpes vulpes*). Dots represent nested PCR-B1-positive cases that could not be genotyped. Genotypes: I (dominant alleles of type I), II (dominant alleles of type II), III (dominant alleles of type III), and P (polymorphic or recombinant strains; $_{RF}$P shows alleles I and III; $_{RD}$P shows alleles I and II; $_{WB}P_{(1)}$ shows alleles I and III; $_{WB}P_{(2)}$ shows alleles III and possible type I). Dotted lines represent the limits of the sampling areas.

nested PCR (B1 gene; Fuentes et al. 2001). B1-positive samples were selected for direct genotyping. *Toxoplasma gondii* genes SAG1, SAG3, GRA6, and BTUB were amplified by multiplex nested PCR (Su et al. 2010) and SAG2 by nested PCR (Howe et al. 1997), and analyzed by restriction fragment length polymorphism (RFLP; Su et al. 2010). Restriction patterns were displayed using 2.5% agarose gel electrophoresis and capillary electrophoresis (Bioanalyzer 2100 apparatus, Agilent Technologies, Inc., Santa Clara, California, USA). Statistical analyses (Fisher's exact test) were performed with R software v.2.12.1 (R Development Core Team 2012).

We detected *T. gondii* DNA in 32.2% of the tissue samples (Table 1). There were no statistically significant differences in prevalence among species except that the prevalence (15%) in wild boar was significantly lower than that in the Spanish ibex

TABLE 1. Prevalence of *Toxoplasma gondii* infection in wild animals in southwestern, Spain by nested PCR-B1.

| Species | *N* | PCR-positive animals | | |
|---|---|---|---|---|
| | | *n* | % | 95% CI[a] |
| Wild boar (*Sus scrofa*) | 61 | 9 | 15 | 7.4–26.7 |
| Red deer (*Cervus elaphus*) | 22 | 4 | 18 | 5.9–41.0 |
| Fallow deer (*Dama dama*) | 21 | 10 | 48 | 26.4–69.7 |
| Mouflon (*Ovies aries musimon*) | 12 | 3 | 25 | 6.7–57.2 |
| Spanish ibex (*Capra pyrenaica victoriae*) | 26 | 12 | 46 | 27.1–66.2 |
| Red fox (*Vulpes vulpes*) | 41 | 21 | 51 | 35.4–66.8 |
| Total | 183 | 59 | 32.2 | 24.6–38.0 |

[a] CI = confidence interval.

or red fox ($P<0.005$). This prevalence was much higher than the 0.7% reported in wild boar in Switzerland by real-time PCR (Berger-Schoch et al. 2011). The 18% we detected in red deer is also higher than the 3% reported for Belgium using real-time PCR (De Craeye et al. 2011). To our knowledge, this is the first report of *T. gondii* DNA in Spanish ibex, fallow deer, and mouflon. Prevalences detected in mouflon and fallow deer were, however, higher than those reported using the mouse inoculation method in France (Aubert et al. 2010) and the Czech Republic (Hejlíček et al. 1997). Our 51% prevalence in foxes was higher than the 30.9% reported for Norway (Prestrud et al. 2008), 18.8% for Belgium (De Craeye et al. 2011), and 15.9% for Germany (Herrmann et al. 2012), on the basis of different PCR analyses. Therefore, we emphasize that the sensitivity of different molecular assays could influence detection of infection, making it difficult to compare prevalences among studies using different methods.

Our direct genotyping methods allowed the characterization, for at least one locus, of 58% of the *T. gondii*-positive samples (Table 2). Due to the limitations of determining *T. gondii* genotype for strains with RFLP patterns at only one or two loci, these strains were classified as "consistent with" type I, II, or III, mixed, or polymorphic presenting different alleles, according to the results. The most common genotype was type II (50.0% of characterized samples), followed by types III (20.6%) and I (8.8%), but mixed infections (11.8%) of types I+III and II+III were detected, as well as recombinant or polymorphic strains (11.8%). Wild boars were infected most frequently by type I and type II. Infections by type II were predominant (81.3%) among the ruminants, and type III was the most commonly found in foxes (45.5%) (Fig. 1).

Type II was the most common genotype; this agrees with that reported in human cases and domestic cats (*Felis catus*) in Spain (Fuentes et al. 2001; Montoya et al. 2008) and for European wild (Aubert et al. 2010; Herrmann et al. 2012) and domestic animals (De Sousa et al. 2006). Type III was highly prevalent, mainly in red foxes, whereas in France, only type II was detected in wildlife (Richomme et al. 2009; Aubert et al. 2010). The high prevalence of type III found in our study also was detected in domestic pigs (*Sus scrofa*; De Sousa et al. 2006) and poultry (Dubey et al. 2006) in neighboring Portugal. Type I was detected in wild boars and foxes, which is a remarkable finding because this genotype is not frequently reported in animals in Europe (Berger-Schoch et al. 2011; De Craeye et al. 2011).

Infections with polymorphic strains, involving alleles from types I and III or alleles from types I and II, were detected. Similar infections have been reported in ruminants and foxes in Europe (Prestrud et al. 2008; Berger-Schoch et al. 2011; Verin et al. 2013). Mixed infections, possibly due to reinfections, were also detected; types I+III and types II+III were also reported previously in sheep (*Ovis aries*), calves (*Bos taurus*), and red foxes from Central Europe (Berger-Schoch et al. 2011; Herrmann et al. 2012). However, in Europe atypical genotypes in wildlife are not usual, whereas in North and South America they are common (Ferreira et al. 2006; Dubey et al. 2011).

TABLE 2. Direct genotyping of *Toxoplasma gondii* DNA detected in tissues from wild game species sampled in southwestern Spain.

| Animal ID[a] | Location (sampling areas) | Tissue | PCR-RFLP genotype (markers)[b] | | | | | Genotype consistent with type[c,d] |
|---|---|---|---|---|---|---|---|---|
| | | | SAG1 | (3′+5′) SAG2 | SAG3 | GRA6 | BTUB | |
| Wild boar J432 | Villuercas–Ibores–Cíjara | Brain | na | na | II | na | na | II |
| Wild boar J462 | Villuercas–Ibores–Cíjara | Brain | na | na | III | I | na | Polymorphic (I/III) |
| Wild boar J476 | Southern sampling areas | Brain | na | na | III | I | na | Polymorphic (I/III) |
| Wild boar J499 | Sierra de San Pedro | Brain | na | na | na | na | II | II |
| Wild boar J528 | Sierra de San Pedro | Brain | I | na | na | I | na | I |
| Wild boar J547 | Southern sampling areas | Brain | I | na | na | I | na | I |
| Wild boar J946 | Sierra de San Pedro | Myocardium | I+III | I or III | na | I+III | I+III | Mixed (I+III) |
| Red deer C263 | Villuercas–Ibores–Cíjara | Brain | na | na | II | I | na | Polymorphic (I/II) |
| Red deer C264 | Villuercas–Ibores–Cíjara | Brain | na | na | II | na | na | II |
| Red deer C322 | Villuercas–Ibores–Cíjara | Brain | II or III | na | III | na | na | III |
| Red deer C348 | Monfragüe National Park | Brain | na | na | na | II | na | II |
| Fallow deer S74 | Sierra de Gredos | Brain | na | na | na | II | II | II |
| Fallow deer S75 | Sierra de Gredos | Brain | na | na | na | II | na | II |
| Fallow deer S76 | Sierra de Gredos | Brain | na | na | na | II | na | II |
| Fallow deer S78 | Sierra de Gredos | Brain | na | na | na | II | na | II |
| Mouflon S81 | Monfragüe National Park | Myocardium | II or III | na | na | II | na | II |
| Mouflon S82 | Monfragüe National Park | Myocardium | na | na | na | II | na | II |
| Mouflon S98 | Sierra de San Pedro | Myocardium | II or III | na | na | II | na | II |
| Spanish ibex S8 | Sierra de Gredos | Brain | na | na | III | III | na | III |
| Spanish ibex S94 | Sierra de Gredos | Myocardium | na | na | na | II | na | II |
| Spanish ibex S104 | Sierra de Gredos | Myocardium | na | na | na | II | na | II |
| Spanish ibex S158 | Sierra de Gredos | Myocardium | na | na | na | II | na | II |
| Spanish ibex S161 | Sierra de Gredos | Myocardium | II or III | na | na | II | na | II |
| Red fox S21 | Monfragüe National Park | Brain | na | II | II | na | na | II |
| Red fox S22 | Sierra de San Pedro | Brain | na | III | II+III | na | II+III | Mixed (II+III) |
| Red fox S26 | Southern sampling areas | Brain | na | na | III | na | na | III |
| Red fox S27 | Southern sampling areas | Brain | na | na | III | na | na | III |
| Red fox S32 | Southern sampling areas | Brain | II or III | III | III | III | III | III |
| Red fox S54 | Sierra de San Pedro | Brain | na | na | III | III | na | III |
| Red fox S55 | Sierra de San Pedro | Brain | II or III | na | III | III | na | III |
| Red fox S83 | Sierra de San Pedro | Brain | I | na | III | na | na | Polymorphic (I/III) |
| Red fox S84 | Sierra de San Pedro | Brain | I | na | I+III | na | III | Mixed (I+III) |
| Red fox S154 | Villuercas–Ibores–Cíjara | Brain | II or III | na | I+III | I+III | I+III | Mixed (I+III) |
| Red fox S155 | Villuercas–Ibores–Cíjara | Brain | II or III | na | II | na | na | II |

TABLE 2. Continued.

| Animal ID[a] | Location (sampling areas) | Tissue | PCR-RFLP genotype (markers)[b] | | | | | Genotype consistent with type[c,d] |
|---|---|---|---|---|---|---|---|---|
| | | | SAG1 | (3′+5′) SAG2 | SAG3 | GRA6 | BTUB | |
| *T. gondii* reference strain RH (type I) | | | I | I | I | I | I | I |
| *T. gondii* reference strain Me49 (type II) | | | II or III | II | II | II | II | II |
| *T. gondii* reference strain C56 (type III) | | | II or III | III | III | III | III | III |

[a] Wild boar = *Sus scrofa*; red deer = *Cervus elaphus*; fallow deer = *Dama dama*; mouflon = *Ovis aries musimon*; Spanish ibex = *Capra pyrenaica victoriae*; red fox = *Vulpes vulpes*.

[b] na = insufficient amplification; RFLP = restriction fragment length polymorphism.

[c] Polymorphic = RFLP patterns consistent with different types detected at different loci.

[d] Mixed infections = RFLP patterns consistent with the presence of multiple types detected at a single locus.

In summary, our results show no distinctive genotype dispersion pattern for the study area, but indicate some differences in the prevalence of types among wild animal species. The genotype distribution of *T. gondii* in southwestern Spain may be more diverse than that indicated by studies in other European countries (De Craeye et al. 2011). It is possible that the small sample size tested in some cases could also diminish the likelihood of detecting landscape patterns. Future studies with a larger number of cases will be of interest.

The circulation and the high prevalence of *T. gondii* infection in wild animals in southwestern Spain may be favored by the wide range of hosts available due to the high biodiversity of the area and the strong environmental persistence of *T. gondii* oocysts due to optimal weather conditions. The red fox, with a small home range (providing detail on local environmental infection) and diet (scavenging and consuming a wide range of prey), may be an appropriate sentinel species for the surveillance of *T. gondii* genotypes and strains (Verin et al. 2013). The genetic variability and the high prevalence of *T. gondii* among the animals we studied and the possible overlap between the wild and domestic cycle of *T. gondii* could have implications for the possible infection of farm animals and humans.

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