Veterinary Parasitology 164 (2009) 296–300

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Genetic characterization of *Toxoplasma gondii* from wild boar (*Sus scrofa*) in France

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ARTICLE INFO

Article history:
Received 23 February 2009
Received in revised form 3 June 2009
Accepted 11 June 2009

Keywords:
Toxoplasma gondii
Wild boar
Sus scrofa
Genotype
Bioassay
PCR–RFLP
Microsatellite
France

ABSTRACT

Toxoplasma gondii strains isolated from domestic animals and humans have been classified into three clonal lineages types I–III, with differences in terms of pathogenicity to mice. Much less is known on *T. gondii* genotypes in wild animals. In this report, genotypes of *T. gondii* isolated from wild boar (*Sus scrofa*) in France are described. During the hunting seasons 2002–2008, sera and tissues of individuals from two French regions, one continental and one insular, were tested for *Toxoplasma* infection. Antibodies to *T. gondii* were found in 26 (17.6%) of 148 wild boars using the modified agglutination test (MAT, positivity threshold: 1:24). Seroprevalence was 45.9% when considering a threshold of 1:6. Hearts of individuals with a positive agglutination (starting dilution 1:6) ($n = 60$) were bioassayed in mice for isolation of viable *T. gondii*. In total, 21 isolates of *T. gondii* were obtained. Genotyping of the isolates using 3 PCR–restriction fragment length polymorphism markers (*SAG1*, *SAG2* and *GRA7*) and 6 microsatellite loci analysis (*TUB2*, *TgM-A*, *W35*, *B17*, *B18* and *M33*) revealed that all belonged to type II lineage. These results underline that wild boar may serve as an important reservoir for transmission of *T. gondii*, and that strains present in wildlife may not be different from strains from the domestic environment.

1. Introduction

The protozoan *Toxoplasma gondii* is a worldwide distributed and obligate intracellular parasite which infects humans and a variety of birds and mammals (Dubey and Beattie, 1988; Tenter et al., 2000). The majority of *T. gondii* isolates from North America and Europe has been classified into three clonal lineages, named types I–III (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). Detailed genetic analysis of each type shows that within-type variation is rare (Grigg et al., 2001), except at the highly polymorphic microsatellite makers (Blackston et al., 2001; Ajzenberg et al., 2002a). In mice, type I strains are

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doi:10.1016/j.vetpar.2009.06.014

considered as virulent, and types II and III as relatively non-virulent. The different studies conducted in humans and domestic animals in France showed a large predominance of only one genotype, type II (Ajzenberg et al., 2002b; Dumètre et al., 2006).

Little is known on the distribution of genotypes of *T. gondii* and on its prevalence in wildlife populations (Dubey et al., 2004a,b, 2007b; Aubert et al., 2008). Moreover, even if acute toxoplasmosis has already been reported in hunters who had consumed uncooked infected meat from wild boar (Choi et al., 1997), little information is available on the risk associated with consumption of wild game meat and on which strains are associated with it. In France, the European wild boar is widely distributed: between 450,000 and 500,000 individuals are hunted each year (Saint-Andrieux and Barboiron, 2009) and meat is consumed by hunters themselves but also distributed locally. In the present study, the seroprevalence of *T. gondii* antibodies in hunted wild boar was investigated in two regions of France and parasite isolation was attempted from seropositive individuals. Isolates were genetically characterized by PCR–RFLP and multilocus microsatellite analysis.

2. Materials and methods

2.1. Wild boar sampling

Animals originated from two distinct regions of France: the Region Champagne-Ardenne (25,606 km^2), in the North-East of the continental France (49°0′N, 4°30′E), and Corsica (8680 km^2), a Mediterranean island (42°9′N, 9°5′E). The climate is continental in Champagne-Ardennes and Mediterranean in Corsica, with annual precipitations nearly equivalent in the two regions (Reims – Champagne-Ardenne 617.8 mm; Ajaccio – Corsica 639.3 mm. Annual means, Meteo France® Data), but minimal and maximal mean annual temperatures higher in Corsica than in Champagne-Ardenne (respectively, 10–20.1 °C, 5.8–14.7 °C). In Champagne-Ardenne, 90% of the sampled animals lived in fenced deciduous forests (Belval private parks and Trois-Fontaines forest); the other 10% were hunted in non-fenced forested plain. In Corsica, all samples came from hunted free wild boar, living in "maquis" (xerophylle vegetation) and non-deciduous mountain forest.

Animals were sampled during the hunting seasons from 2002 to 2007 in Champagne-Ardenne, and during the hunting season 2007–2008 in Corsica, thanks to some hunters and the opportunities of collecting biological material from other research or surveillance programs.

Blood samples from hunted animals were collected from the heart or the thoracic cavity using a syringe. Hearts were collected and placed into a plastic container with sterile saline (0.9% [w/v] NaCl) containing 120 U/mL penicillin-G and 120 μg/mL streptomycin. Hearts and sera were kept refrigerated (4 °C) during 1–5 days and transported to the laboratory of the National Reference Centre for toxoplasmosis in Reims, France. Age, gender and place of hunting were recorded for each animal. Age was determined based on coat colour and estimated body weight, and each individual was classified either as juvenile (0–1 year) or adult (>1 year).

2.2. Serologic examination

Fluid samples were tested for antibodies to *T. gondii* with the modified agglutination test (MAT) as previously described (Dubey and Desmonts, 1987). MAT is the most sensitive and specific test for the serodiagnosis of toxoplasmosis in swine (Dubey, 1997b). Sera were diluted two-fold starting at a 1:6 dilution and screened until a dilution of 1:12,800. Positive and negative controls were included in each test. In accordance with previous studies, sera with an agglutination titer of 1:24 or higher were first considered as positive. We also estimated seroprevalence using a threshold titer of 1:6 because individuals with low titers often revealed infection (see Section 3).

2.3. Bioassay for T. gondii infection

Hearts of individuals with a agglutination reaction (starting at a 1:6 dilution) were bioassayed in outbreed female Swiss Webster mice (Charles River Laboratory, France) (Villena et al., 2004). All mice were first confirmed to be seronegative for *T. gondii*. Whole heart, or 200 g of heart was mixed and incubated at 37 °C during 2.5 h with trypsin (final concentration 0.25%). The suspension was then filtered, centrifuged, washed and suspended in saline solution containing penicillin-G and streptomycin. The homogenate was inoculated intraperitoneally to 3–6 mice, depending on the volume of the centrifugation pellet. Mice were tested for seroconversion with the MAT (1:24 dilution) 4 weeks postinoculation (pi) and finally sacrified 60 days pi. Tissue cysts in brains of seropositive mice were detected by microscopic examination.

2.4. Genetic characterization for T. gondii

Brain cysts from seropositive mice were isolated by percoll gradient centrifugation and DNA was extracted using a QIAamp DNA minikit (Qiagen, Courtaboeuf, France). Strain typing was performed using 3 PCR–restriction fragment length polymorphism (RFLP) markers (*SAG1*, *SAG2*, *GRA7*). Genetic characterization was also performed at 6 microsatellite loci in a multiplex PCR assay (TUB2, TgM-A, W35, B17, B18 and M33) (Ajzenberg et al., 2005; Dubey et al., 2007a).

2.5. Statistical analysis

Seroprevalence was estimated using both 1:24 and 1:6 thresholds, and its 95% confidence interval was calculated. Comparisons of gender and age distributions in the two regions were performed using Chi-square tests. In order to compare seroprevalence in the two regions, age-adjusted seroprevalences were calculated (Dohoo et al., 2003). All analyses were performed using R (R Development Core Team, 2008). The differences were considered statistically significant when $p < 0.05$.

Table 1
Age-adjusted serorevalence of *Toxoplasma gondii* antibodies and agglutination titers obtained by MAT in wild boars from Champagne-Ardenne and Corsica, France.

Animals	No. sampled	MAT agglutination titer				Total positive	Positive[a]/tested		Prevalence (%)[b] ± SE 95% CI
		0	6	12	≥24[c]		Juvenile	Adult	
Champagne-Ardenne	104	55	8	20	21	21	11/49	7/38	20.1 ± 8.7
Corsica	44	25	6	8	5	5	1/6	4/38	13.1 ± 13.8
France (Overall)	148	80	14	28	26	26	12/55	11/76	17.6 ± 6.1

[a] MAT ≥ 24.
[b] Prevalence standardised on age.
[c] Maximal titer observed: 3200.

3. Results

A total of 148 wild boars were sampled, 104 in Champagne-Ardenne and 44 in Corsica. Age and gender were available for 131 samples. The demographic structure of the samples differed, with more juveniles than adults in the Champagne-Ardenne (49 versus 38), and reverse structure in Corsica (6 juveniles, 38 adults) (Chi-square test, $p < 0.001$). The sex-ratio for the 2 areas was not statistically different (Champagne-Ardenne 1.4 males/female versus Corsica 1.2, Chi-square test, $p = 0.796$).

Antibodies to *T. gondii* (MAT, titer ≥ 1:24) were found in 26 of the 148 animals, indicating an overall seroprevalence of 17.6% (95% CI: 11.5–23.7%) at threshold 1:24. Considering a threshold titer of 1:6, seroprevalence was 45.9% (95% CI: 37.9–54.0%). Titer of *T. gondii* antibodies and details per age class for each sampling region are shown in Table 1. After adjusting on age, seroprevalences in Champagne-Ardenne (20.1 ± 8.67%) and in Corsica (13.1 ± 13.8%) were not significantly different ($p = 0.477$).

Among the 68 hearts from individuals with MAT titers of 1:6 or higher, 60 were bioassayed in mice. Sixteen bioassays were not conclusive because none of the mice survived after 48 h pi. *T. gondii* was isolated from 21 of the 44 remaining hearts (47.7%, Table 2). Noteworthy, for 22 samples from Champagne-Ardenne, one of the inoculated mice died of presumed bacterial infections during the first 3 days pi, in spite of additional antibiotic treatment; these mice were not examined for *T. gondii* infection. Mortality in mice due to a virulent strain usually occurs after 7 days pi (Dubey et al., 2008). Thus, here, all mice mortalities were attributed to bacterial infections, which are frequent with samples from wild species (Aubert, pers. comm.). The parasite was isolated from 11 (45.8%) of 24 individuals with MAT titer of 1:6 and 1:12, and from 10 of 20 (50.0%) with titer of 1:24 or higher. Genotyping of the 21 *T. gondii* isolates using the 3 PCR–RFLP markers and the 6 microsatellite markers revealed a type II genotype for all isolates (Table 2).

4. Discussion

The MAT titers of 1:20–1:25 have been validated to detect IgG antibodies to *T. gondii* in sera of domestic pigs (Dubey et al., 1995). Using this cut-off, we found antibodies in 17.6% of the tested wild boars. However, individuals with an agglutination titer lower than 1:25 actually carried *Toxoplasma* cysts. These results raise the question of the choice of the cut-off when interpreting a serological test, especially in wildlife when using results from tests developed for domestic species. Here, we advocate that 1:6 is a biologically relevant threshold since many individuals with 1:6 titer actually carried *Toxoplasma* cysts. None of the samples without agglutination reaction were bioassayed, because a previous study in sheep showed that none of 307 seronegative samples revealed infection at bioassay (Halos et al., 2008). However, given the low level of antibodies in some individuals, seronegative individuals may be bioassayed, or serological tests could be performed using lower dilutions.

Even if our sample is not representative of the national situation, our results indicate a high level of exposure of *T. gondii* among wild boar, both in a continental and an insular region of France. Several previous studies already showed that toxoplasmosis is common in wild boars in Europe: seroprevalence was estimated at 19% in Austria (indirect immuno-fluorescence test, $n = 269$, Edelhofer et al., 1996), 15% in the Czech Republic (Dye Test, threshold 1:4, $n = 124$, Hejlicek et al., 1997) or 26.2% (indirect fluorescence antibody test, $n = 565$, Bártová et al., 2006) in the Czech Republic and 8.1% in the Slovak Republic (commercial ELISA, $n = 320$, Antolová et al., 2007). Most of these seroprevalences fall in the range of values of our study (17.6–45.9% depending on the threshold used). However, they are not always comparable since distinct methods or thresholds are used. The only studies considering MAT 1:25 found antibodies to *T. gondii* in wild boar in 31% and 37% ($n = 108$ and 149, Diderrich et al.,

Table 2
Genotyping of *Toxoplasma gondii* isolated in wild boars from Champagne-Ardenne and Corsica, France.

Animals	No. sampled	No. seropositive[a]	No. bioassayed[b] (analysed[c])	Isolates (%[d])	Genotype
Champagne-Ardenne	104	49	41 (26)	13 (50.0)	II
Corsica	44	19	19 (18)	8 (44.4)	II
France (Overall)	148	68	60 (44)	21 (47.7)	II

[a] Starting at a 1:6 dilution.
[b] 8 hearts were not bioassayed because of technical reasons.
[c] 16 bioassays were not analysed for cysts detection because all mice died between days 1 and 3 pi (15 from Champagne-Ardennes, 1 from Corsica).
[d] Isolates/number analysed.

1996) and in 18% (n = 174, Dubey et al., 1997), in the U.S.A., and in 38.4% in Spain (n = 507, Gauss et al., 2005). In this last study, the authors noted strong variations among sampling sites, probably associated with variation in contamination of the environment by cats, different climatic conditions that may have affected the survival of oocysts, management and population densities of wild boar. Here, in two regions comparable for rainfall but different in their temperatures and vegetation, seroprevalences were non-significantly different.

All isolates were found to belong to the type II lineage. In France, type II is largely predominant in human congenital toxoplasmosis (Ajzenberg et al., 2002b). Moreover, only type II strains have been isolated from domestic animals (Dumètre et al., 2006) or wild birds (Aubert et al., 2008). Due to the low number and non-representative geographic distribution of samples analysed, we cannot preclude the hypothesis that other genotypes are also present in wildlife in France. However, genotype II is probably dominant in wild boar, thus no specific strain is expected if transmission from wild boar to humans occurs.

Consumption of raw or undercooked meat is the main route of infection in humans in Europe, representing 30–63% of infections depending on the country considered (Cook et al., 2000). Specifically, pork meat was considered as one of the major sources (Dubey and Beattie, 1988). Over the last two decades, however, infection in pigs decreased notably with changes in pig production and management (Tenter et al., 2000), which raise all the more the question of the role of other species, including game, as sources of human infection. Traditionally, wild boar meat is consumed after a long cooking, which kills tissue cysts (Dubey, 2000). But food habits tend to change with consumption of raw meat (barbecue). Specifically, in Corsica, raw and salted pork meats products (“salsiccia”, “figatelli”) are traditionally prepared, and can contain wild boar meat (Casabianca, 1996). The curing of meat does not affect the parasite immediately and the survival time of tissue cysts varies with the concentration of the salt solution and the temperature of storage (Dubey, 1997a). Finally, salting does not kill all tissue cysts in home-made pork sausages in Tenter et al. (2000). Although the origins of most human infections cannot be documented precisely, acute toxoplasmosis has been reported in hunters after consumption of undercooked meat from wild pigs (Choi et al., 1997). Thus, while prevalence of *T. gondii* in pork meat decreases, wild game meat should not be neglected as a significant and possibly increasing source of toxoplasmosis.

Acknowledgments

We thank the technical staff of ONCFS (Office National de la Chasse et de la Faune Sauvage) of Trois-Fontaines, C. De Gevigney (Parc de Belval), and hunters from Champagne-Ardennes, especially H. Bertrand, and from Corsica, especially O. Maestrini, for their help in obtaining samples. We thank R. Geers and N. Ortis for helpful technical assistance. This work was supported by Programme Bioscope (ANR 05 SEST 048-02) from the French National Research Agency.

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