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Spread and genotyping of Toxoplasma gondii in naturally infected Alpine chamois (Rupicapra r. rupicapra)

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Abstract:	The complex life-cycle of Toxoplasma gondii involves many animal species, raising zoonotic, economic, and conservation issues. This complexity is reflected in the molecular structure of T. gondii, whose different genotypes differ in pathogenicity. Among the intermediate hosts of T. gondii, wild ungulates may be a source of human infection. Despite intense hunting activity and the consumption of raw or undercooked meat, little information is available on the spread of T. gondii and the distribution of its genotypes in these species, including the alpine chamois (Rupicapra r. rupicapra). Ninety-three sera and 50 brain tissues from chamois were sampled (1) to investigate the spread of T. gondii with serological and molecular analyses, and (2) to genotype the strains with a restriction fragment length polymorphism analysis of the SAG2 locus. The prevalence of T. gondii was low on both serological (3.2%) and molecular (2%) analyses, and infections were concentrated in individuals > 1 year old. These findings demonstrate the sporadic presence of the protozoan in this species on consistent diagnostic tests. Horizontal transmission seems to be the main route of infection, and cats are the only definitive host in the study area. This prevalence suggests that the environment of the chamois is less contaminated with oocysts than environments close to human settlements. The SAG2 type II genotype was detected in this species for the first time. Although this genotype is predominant in human toxoplasmosis, these results suggest that the chamois is a minor source of human infection.
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Spread and genotype of *Toxoplasma gondii* in naturally infected Alpine chamois (*Rupicapra r. rupicapra*)

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Abstract

The complex life-cycle of *Toxoplasma gondii* involves many animal species, raising zoonotic, economic, and conservation issues. This complexity is reflected in the molecular structure of *T. gondii*, whose different genotypes differ in pathogenicity. Among the intermediate hosts of *T. gondii*, wild ungulates may be a source of human infection. Despite intense hunting activity and the consumption of raw or undercooked meat, little information is available on the spread of *T. gondii* and the distribution of its genotypes in these species, including the alpine chamois (*Rupicapra r. rupicapra*). Ninety-three sera and 50 brain tissues from chamois were sampled (1) to investigate the spread of *T. gondii* with serological and molecular analyses, and (2) to genotype the strains with a restriction fragment length polymorphism analysis of the *SAG2* locus. The prevalence of *T. gondii* was low on both serological (3.2%) and molecular (2%) analyses, and infections were concentrated in individuals > 1 year old. These findings demonstrate the sporadic presence of the protozoan in this species on consistent diagnostic tests. Horizontal transmission seems to be the main route of infection, and cats are the only definitive host in the study area. This prevalence suggests that the environment of the chamois is less contaminated with oocysts than environments close to human settlements. The *SAG2* type II genotype was detected in this species for the first time. Although this genotype is predominant in human toxoplasmosis, these results suggest that the chamois is a minor source of human infection.

Keywords: Toxoplasmosis; wild ungulates; PCR–RFLP; genotypes; zoonotic risk.

Introduction

The complex life cycle of *Toxoplasma gondii* involves a broad spectrum of intermediate host species (Dubey 1992; Conrad et al. 2005; Bártová et al. 2006; Ryser-Degiorgis et al. 2006; Sadrebazzaz et al. 2006; Sobrino et al. 2007; Jokelainen et al. 2010; Lopes et al. 2011) that have differential susceptibility and receptivity to the infection. *Toxoplasma gondii* represents a public health concern (Saki et al. 2013), being the most common among parasitic zoonoses (EFSA 2007). In the livestock industry, the impact of *T. gondii* on reproductive performance causes economic losses (Huong et al. 1998; Duncanson et al. 2001; Masala et al. 2003; Pereira-Bueno et al. 2004; Morley et al. 2008; Rossi et al. 2011). Further, fatal cases of *T. gondii* infection have been reported in several wild species (Jokelainen et al. 2011, 2012; Jokelainen and Nylund 2012; Jokelainen and Vikøren 2014), raising conservation issues. These concerns should also be considered in relation to the three major multilocus genotypes of *T. gondii* (types I, II, and III), which differ in their virulence, as reported in murine models (FAO/WHO 2014). Type I strains are uniformly lethal, whereas types II and III are avirulent or significantly less virulent than type I (Robert-Gangneux and Dardé 2012). All the *T. gondii* genotypes occur in Europe (Richomme et al. 2009; Herrmann et al. 2012; Burrells et al. 2013). The roles of the *T. gondii* genotypes in its virulence in humans are still contentious, but the genetic and immune status of the host appear to influence the virulence of these strains (Robert-Gangneux and Dardé 2012). Therefore, the spread of *T. gondii* and the distribution of its genotypes must be investigated in animals that may be sources of human infection, including wild ungulates. In this context, several species have been reported as intermediate hosts of *T. gondii* (Kapperud 1978; Hejlíček et al. 1997; Vikøren et al. 2004; Magnino et al. 2011; Formenti et al. 2015), and the zoonotic risk is related to consumption of raw or undercooked meat (Dubey and Beattie 1988; Gauss et al. 2006; Park et al. 2011), or to the handling of their carcasses (McDonald et al. 1990; Dubey 1994; Kapperud et al. 1996; Tenter et al. 2000). Thus, zoonotic potential should be evaluated in terms of the amount of game meat harvested every year (Ross et al. 2001; Gamarra et al. 2008; Kijlstra and Jongert 2008). In particular, species that are subject to intense hunting activity and are consumed undercooked or even raw (typically local products), such as alpine chamois (*Rupicapra r. rupicapra*) (Gaviglio et al. 2015), warrant particular attention. Although around 12,600 chamois are harvested in the Italian Alps in each hunting season, with the consequent availability of over 180 tons of meat (Ramanzin et al. 2010), little information is available on the spread of *T. gondii* and none on the distribution of its genotypes in this species. Therefore, we performed an epidemiological study of *T. gondii* in chamois in the Central Italian Alps (1) to investigate the spread of infection using serological and molecular techniques, and (2) to genotype the protozoan strains with a restriction fragment length polymorphism (RFLP) analysis of the *SAG2* locus.

Materials and methods

Study area

The study area lies in the northeasternmost part of Piedmont in the Central Italian Alps (province of Verbania: 46°07′N, 8°17′E) at altitudes ranging from 250 m asl to 3272 m asl, and spanning an area of 72,740 ha. The characteristics of the area are particularly favorable for chamois, which is the most numerous of the wild ungulates here. This species is distributed over an area of 34,265 ha and the annual census showed a mean value of 6.6 individuals/100 ha.

Sampling

Serological and molecular samples were gathered from alpine chamois during, respectively, three (2011-2013) and two (2011-2012) hunting seasons. In particular, brain tissue was selected for the detection of *T. gondii* in PCR since this organ is the most frequently involved during Toxoplasmosis and, post infection, the protozoan may remain in brain tissue throughout life (Robert-Gangneux and Dardé 2012). Moreover, the brain has been previously used for *T. gondii* molecular analysis in both domestic (Masala et al. 2003; Asgari et al. 2011; Dubey et al. 2011) and wild animals (Dubey et al. 2004; Hůrková and Modrý 2006; De Craeye et al. 2011), revealing reliable test sensitivity. Blood samples were collected in the field directly from the major blood vessels by the hunters during the bleeding of carcasses. In accordance with Italian Law (157 of 11/02/1992), hunters must carry all culled wild ungulates to control centers, where the age, sex, shooting site (valley, altitude, exposure, etc.), and morphobiometric measurements of each animal are registered. Hunters, who voluntarily agreed to participate in the study, permitted us to gather brain tissue samples during the post-mortem inspection of the carcasses. In particular, we collected brain tissue samples from chamois in suitable biological conditions by removing the obex, as is usually done to detect prion diseases in domestic animals (Langeveld et al. 2003). Moreover, after centrifugation of blood samples, sera were screened to exclude from serological analyses those with an unsuitable level of hemolysis. A total of suitable 93 sera and 50 brain tissue samples (Table 1) were collected from 133 chamois, since overall 10 chamois were sampled for both serological and molecular investigations. All the sera and brain samples were immediately frozen and stored at –20 °C.

Serological investigation

Toxoplasma gondii antibodies were detected in the sera with a commercial enzyme-linked immunosorbent assay (ID Screen® Toxoplasmosis Indirect ELISA, IDVET, Montpellier, France), validated for ruminants, with a reported sensitivity of 91.3% and specificity of 98.7% (Chong et al. 2011). The test was performed according to the manufacturer's instructions.

Molecular investigation

Detection of T. gondii with PCR

DNA was extracted from the chamois brain samples with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. All 50 brain samples were assayed with PCR using the primers described by Homan et al. (2000), TOX4 (5′-CGCTGCAGGGAGGAAGACGAAAGTTG-3′) and TOX5 (5′-CGCTGCAGACACAGTGCATCTGGATT-3′), which target a 529-bp noncoding region (protocol 1). A second PCR protocol (Hurtado et al. 2001) (protocol 2) was used to confirm all positive or weak/doubtful results. Protocol 2 consisted of two successive amplifications performed in a single tube with the following primers: the external primers were NN1 (5′-CCTTTGAATCCCAAGCAAAACATGAG-3′) and NN2 (5′-GCGAGCCAAGACATCCATTGCTGA-3′), and the internal primers were Tg-NP1 (5′-GTGATAGTATCGAAAGGTAT-3′) and Tg-NP2 (5′-ACTCTCTCTCAAATGTTCCT-3′), which target the 18S–5.8S rRNA internal transcribed spacer 1 (ITS1). A third PCR–RFLP protocol, reported by Magnino et al. (1998) (protocol 3), was used to confirm the positive results and to exclude any possible cross-reaction with *Neospora caninum* or *Sarcocystis* spp. because the primers used identify all three protozoa. Primers APIF (5′-AAGTATAAGCTTTTATACGGC-3′) and APIR (5′-CACTGCCACGGTAGTCCAATAC-3′) target the 18S small subunit ribosomal gene of *T. gondii*. The amplified products were digested with the *Bse*DI restriction enzyme.

Genotyping analysis

The *T. gondii* genotypes (I, II, or III) were determined by characterizing the *SAG2* gene using PCR-based RFLP (Howe et al. 1997; Fuentes et al. 2001; Gallego et al. 2006; Pezerico et al. 2009). Two nested PCRs were performed separately to amplify the 5′ and 3′ ends of this gene: the 5′ end of the locus was amplified with the primers SAG2.F4 (5′-GACCTCGAACAGGAACAC-3′) and SAG2.R4 (5′-GCATCAACAGTCTTCGTTGC-3′) and the second amplification was performed with the internal primers SAG2.F (5′-GAAATGTTTCAGGTTGCTGC-3′) and SAG2.R2 (5′-GCAAGAGCGAACTTGAACAC-3′). The amplified products were digested with *Sau*3AI.

The 3′ end of the locus was similarly analyzed with the primers SAG2.F3 (5′-TCTGTTCTCCGAAGTGACTCC-3′) and SAG2.R3 (5′-TCAAAGCGTGCATTATCGC-3′) for the initial amplification and the internal primers SAG2.F2 (5′-ATTCTCATGCCTCCGCTTC-3′) and SAG2.R (5′-AACGTTTCACGAAGGCACAC-3′) for the second round of amplification. The amplification products were digested with *Hha*I. All the restriction fragments were analyzed with 2% agarose gel electrophoresis.

Results

The serological investigation showed a *T. gondii* prevalence of 3.2% (3/93; 95% confidence interval [CI]: 0.37–6.8). The positive sera were from a 1-year-old female and two five and fifteen year old adult males.

The molecular analysis of brain tissue detected the protozoan DNA in a six-year-old male, indicating an overall prevalence of 2% (1/50; 95% CI 1.88-5.88). *Toxoplasma gondii* DNA was detected with all three PCR protocols (Magnino et al. 1998; Homan et al. 2000; Hurtado et al. 2001), excluding any possible cross-reaction with *N. caninum* or *Sarcocystis* spp. Genetic characterization of the isolated DNA revealed the *SAG2* type II genotype.

Discussion

This epidemiological survey of the distribution of *T. gondii* in chamois detected the presence of the *SAG2* type II genotype and an overall prevalence of 2%–3.2%, which supports the sporadic presence of the protozoan in this species. In the population studied, the *T. gondii* seroprevalence (3.2%) was low, indicating the occasional presence of the protozoan in this species. The three seropositive sera were recorded in individuals > 1 year of age, and if we exclude a spurious result arising from the limited sample size of kids (n = 2), this finding supports the hypothesis that horizontal transmission is the main route of infection. This result implies that feral and semidomestic cats are the principal source of environmental contamination (shedding infecting oocysts) because, the only other definitive host, the lynx (*Lynx lynx*), is sporadic in the Central Italian Alps. Based on these considerations, two alternative hypotheses emerge. The first proposes that the low seroprevalence of *T. gondii* results from the limited exposure of the chamois to infective oocysts, ascribed to its use of remote high-altitude habitats, because cats usually live close to human settlements and in built-up areas. According to the second hypothesis, the low susceptibility of this intermediate host to *T. gondii* infection cannot be ruled out. However, Gotteland et al. (2014) reported a seroprevalence of 16.8% in chamois from an area in the French Alps in which European wildcats (*Felis silvestris silvestris*), lynx, and domestic cats have been observed. Marco et al. (2009) also demonstrated the susceptibility of *Rupicapra pyrenaica* to *T. gondii* infection, recording the death of an individual with systemic toxoplasmosis and Gram-negative sepsis. These considerations support our first hypothesis. *Toxoplasma gondii* DNA was detected in this species for the first time with all three PCRs performed. This molecular positivity emerged in an individual for which serum sample was not collected by hunter due to difficulties in field sampling of alpine chamois. Likewise at the control center we were not able to gather the brain tissue sample of the three seropositive subjects. Therefore, this fact prevented us to extend the genotyping analysis to these individuals. On those 10 chamois for which both serum and brain tissue were sampled, both serology and PCR gave a negative result. The fact that both serological and molecular analysis were performed on a limited sample size (n=10) prevented us to statistically estimate the agreement between diagnostic tests. However, the serological (3.2% prevalence) and molecular

(2% prevalence) analyses showed consistent results suggesting that these approaches were able to reveal the epidemiological conditions of the studied population. Furthermore these results were also consistent with previous Italian findings (Gennero et al. 1993; Gaffuri et al. 2006; Ferroglio et al. 2014).

The genotyping analysis revealed the presence of *T. gondii SAG2* type II. This is the first report of a *T. gondii* genotype in wild Italian ungulates. Despite having used different protocols, Verin et al. (2013) demonstrated the occurrence of polymorphic strains (combination of different type I and III alleles) in red foxes (*Vulpes vulpes*) and Mancianti et al. (2013, 2014) detected genotypes I and III and atypical genotypes (with hints of genotype I) in goats and genotypes II and III in donkeys. Therefore, all the *T. gondii* genotypes appear to be present in Italy. The genotyping protocol we used here is useful because it is highly sensitive and only small amounts of DNA are required (Howe et al. 1997; Gallego et al. 2006). Moreover, the gene investigated is ideally suited for rapid genotyping, containing multiple lineage-specific polymorphisms, and is therefore useful for screening large numbers of samples (Howe et al. 1997; Gallego et al. 2006). However, the limitation of a single-locus analysis is that it cannot detect recombination events or atypical alleles (Dumètre et al. 2006; Berger-Schoch et al. 2011), which could be even more pathogenic than the other three clonal types (FAO/WHO 2014).

Although *T. gondii* type II is predominant in human toxoplasmosis (Gallego et al. 2006; FAO/WHO 2014), the detection of its DNA in chamois brain tissue does not allow the actual zoonotic risk to be estimated because the corresponding number of cysts in the skeletal muscles is still unknown. Moreover, if we extrapolate our recorded prevalence to all the chamois hunted each year in Italy, around 250 infected individuals are consumed each year. Therefore, among game species, the chamois does not appear to be a principal source of human toxoplasmosis, but zoonotic infections associated with the consumption of raw or undercooked meat or the handling of infected carcasses cannot be ignored.

Conclusions

Toxoplasma gondii of the *SAG2* type II genotype was detected in chamois for the first time. The consistent prevalence detected with serological and molecular analyses supports the sporadic presence of the protozoan in this species. This finding is probably attributable to the use of remote habitats by the chamois. Because atypical genotypes are more virulent and genetic recombination occurs more frequently in wildlife species than domestic species (Ajzenberg et al. 2004; Grigg and Sundar 2009; De Craeye et al. 2011), more genetic markers should be investigated. However, the chamois does not seem to be a major source of human toxoplasmosis, although the zoonotic risk associated with the consumption of raw or undercooked meat or the handling of infected carcasses cannot be ignored.

Compliance with Ethical Standards

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Conflict of Interest: The authors declare that they have no conflicts of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study did not include any experiments involving human participants.

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Table 1 Overall chamois sample size according to age classes

Age class	Serum samples	Brain tissue samples
Kid	2 (2.2%)	0 (0%)
Yearling	38 (40.9%)	21 (42.0%)
> 2 years old	52 (55.9 %)	29 (58.0%)
Not determined	1 (1.1%)	0 (0%)
Total	93	50