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Research paper

Circulating genotypes of *Toxoplasma gondii* in Northwestern Italy

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ABSTRACT

Toxoplasma gondii is an apicomplexan parasite that in Europe is genetically characterized by three main clonal genotypes, with a lesser prevalence of atypical patterns. Data on the genotypes of *T. gondii* circulating both in wildlife and livestock in Northern Italy are scarce. In the present study skeletal muscle samples of cattle (*Bos taurus*), swine (*Sus scrofa domesticus*), fox (*Vulpes vulpes*), roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*) were genotyped by using a nested PCR of 6 loci (alt.SAG2, GRA6, 5′SAG2, BTUB, C22-8 and SAG1) and in silico RFLP. High prevalence of genotype I and non-canonical genotypes were observed in this study, with some differences in the population structure of the parasite between livestock and wildlife. Genetic variability of *T. gondii* in Europe could be more variable than previously thought, with possible implication for public health.

1. Introduction

Toxoplasma gondii is an obligate intracellular parasitic protozoan belonging to the phylum Apicomplexa, representing a potential hazard for human health. Human toxoplasmosis is spread worldwide with a seroprevalence that ranges between 22.5% and 75% (Montoya and Liesenfeld, 2004), and is considered the second leading cause of death among foodborne pathogens in United States (Jones et al., 2014). *T. gondii* is a zoonotic parasite which has two types of hosts: the definitive host is a feline, while a wide range of warm blooded animals can act as intermediate hosts including men.

Humans can get infected by *T. gondii* through different transmission routes: (i) food-borne transmission; (ii) vertical transmission; (iii) transplants and transfusions transmission. Food-borne transmission can occur by ingestion of water and vegetables contaminated by sporulated oocysts (Torrey and Yolken, 2013), by ingestion of raw or undercooked meat containing bradyzoites (Schluter et al., 2014) and finally by consuming unpasteurized milk or fresh cheese made with raw milk containing tachyzoites (Mancianti et al., 2013). Primary infection of *T. gondii* in pregnant women can lead to congenital toxoplasmosis in foetuses and newborns following the dissemination of tachyzoites through the placenta (McAuley, 2014).

While human toxoplasmosis in immunocompetent adults is mainly asymptomatic or characterized by mild symptoms like cervical lymphadenopathy, fever, malaise and myalgia (Weiss and Dubey, 2009), *T. gondii* has been recently associated with different central nervous system disorders like epilepsy (Palmer, 2007) and schizophrenia (Torrey et al., 2007), and with an increased risk of car accidents (Flegr et al., 2002).

Although risk factors for *T. gondii* infection are different based on the country and the socioeconomic status, in Europe the meat-borne transmission seems to be one of the most frequent infection routes (Cook et al., 2000). The prevalence of this parasite in livestock differs according to the considered species and management systems. Concerning species, cattle has been considered an unsuccessful host for *T. gondii,* for its ability to control infection by reducing or eliminating the parasite (Dubey and Jones, 2008). However, a serological survey showed antibodies against *T. gondii* in up to 92% of cattle (Avezza et al., 1993), while a meta-analysis study showed a prevalence that ranges between 0 and 2.6% in beef (Belluco et al., 2016). Swine, sheep and goat are the most infected species (Tenter et al., 2000), with an average prevalence of 12.3%, 14.7% and up to 26% respectively (Belluco et al., 2016). Management system of livestock has been considered a risk factor for *T. gondii* infection. Organically raised pigs, for instance, have showed a significant higher seroprevalence than conventionally raised pigs, because this “animal-friendly” management system allows animals to come in contact with source of *T. gondii* like contaminated soil, grass, water and infected rodents and birds (Kijlstra and Jongert, 2008).

Recently, the population of wild animals has increased in some parts of Europe – especially red deer, roe deer and wild boar (Ramanzin et al., 2010) – and consequently also the popularity of game meat. Consumption of game meat has been associated with an increased risk of congenital toxoplasmosis in pregnant women (Cook et al., 2000) and was the cause of several outbreaks of toxoplasmosis (Ross et al., 2001). Furthermore, Ferroglio et al. (2014) have observed that the prevalence of *T. gondii* in wild animals was different according to feeding habits

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(carnivores and omnivores showed higher prevalence than herbivores for the possible infection through infected prey) and to habitat types (in chamois and red deer authors recorded a lower prevalence than in roe deer).

Particularly in Europe and North America, genetic structure of *T. gondii* has been considered mainly clonal, with 3 distinct genotypes (type I, II and III) (Boothroyd and Grigg, 2002). Recently, a fourth clonal genotype has been observed in USA (Khan et al., 2011). In South America, however, the genetic structure of the parasite is more heterogeneous with the presence of many non-clonal atypical genotypes (Shwab et al., 2014).

In Northern Italy, information on circulating *T. gondii* genotypes are scarce, so the aim of this study was to evaluate the distribution of the genotypes occurring in domestic and wild animals in Northwest Italy.

2. Materials and methods

Overall, sixty-five *T. gondii*-positive skeletal muscle samples were used for this genotyping study. These samples were previously tested for *T. gondii* positivity by using PCR targeting the 200–300 fold repetitive 529 bp DNA fragment (Bosio et al., 2013; Ferroglio et al., 2014). Samples derived from cattle (*Bos taurus*) (n = 11), swine (*Sus scrofa domesticus*) (n = 15), fox (*Vulpes vulpes*) (n = 18), roe deer (*Capreolus capreolus*) (n = 3) and wild boar (*Sus scrofa*) (n = 18). Wild animals derived from hunting activities or were found dead between October 2009 and December 2012. Fig. 1 showed the places in which samples were collected. Samples were processed for *T. gondii* genotyping using a nested PCR of 6 RFLP markers: alt.SAG2, GRA6, 5′SAG2, BTUB, C22-8 and SAG1. Primers (previously described by Su et al., 2010), together with the annealing temperatures, are summarized in Table 1. PCR reaction for Step I was carried out in a final volume of 25 µl containing 12.5 µl of PCR Master Mix (Promega, Madison, WI), 4 pmol of each external primer and 4 µl of target DNA. The thermal cycler protocol was 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 2 min, followed by a step of 72 °C for 10 min. The I step product was used as template for step 2 reaction. Briefly, 12.5 µl of PCR Master Mix (Promega, Madison, WI), 8 pmol of each internal primer and 3 µl of template were used. The thermal cycler protocol was 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1.30 min, and finally 72 °C for 10 min. DNA extracted from *T. gondii* genotype I, II and III cell cultures was used as positive control and amplified during each PCR step, together with a negative control.

All the PCR products were sent for sequencing (Macrogen Europe, The Netherlands) and in silico digested for all the markers by using the free online software NEBCutter (New England Biolabs Inc; http://tools.neb.com/NEBcutter2/index.php) (Vincze et al., 2003).

Results were statistically evaluated by Fisher exact test or by Chi square test depending on the expected prevalence, by using the R software (R Development Core Team, 2015).

3. Results

Out of 65 samples tested, we obtained sequences that allow us to genotype 43 of them (6 bovines, 15 pigs, 7 foxes, 1 roe deer and 14 wild boars). The nucleotide sequences obtained in this study are available in GenBank™ (accession number from MG587956 to MG588017). Only for one sample it was possible to obtain the amplification for all the inquired markers, while most of the samples were genotyped at less than 6 alleles because PCR amplifications failed. Results are summarized in Table 2. Thirty-one samples out of 43 showed Type I alleles at least at one locus (p = 72.09%; CI95% 57.31%–83.25%), while Type II and III alleles were present in two (p = 4.65%; CI95% 1.28%–15.46%) and three (p = 6.98%; CI95% 2.40%–18.61%) samples respectively. Seven samples (p = 16.28%; CI95% 8.12%–29.97%) showed mixed Type alleles. Furthermore, a higher prevalence of genotype I was recorded in omnivorous domestic species (pigs) than in carnivorous and

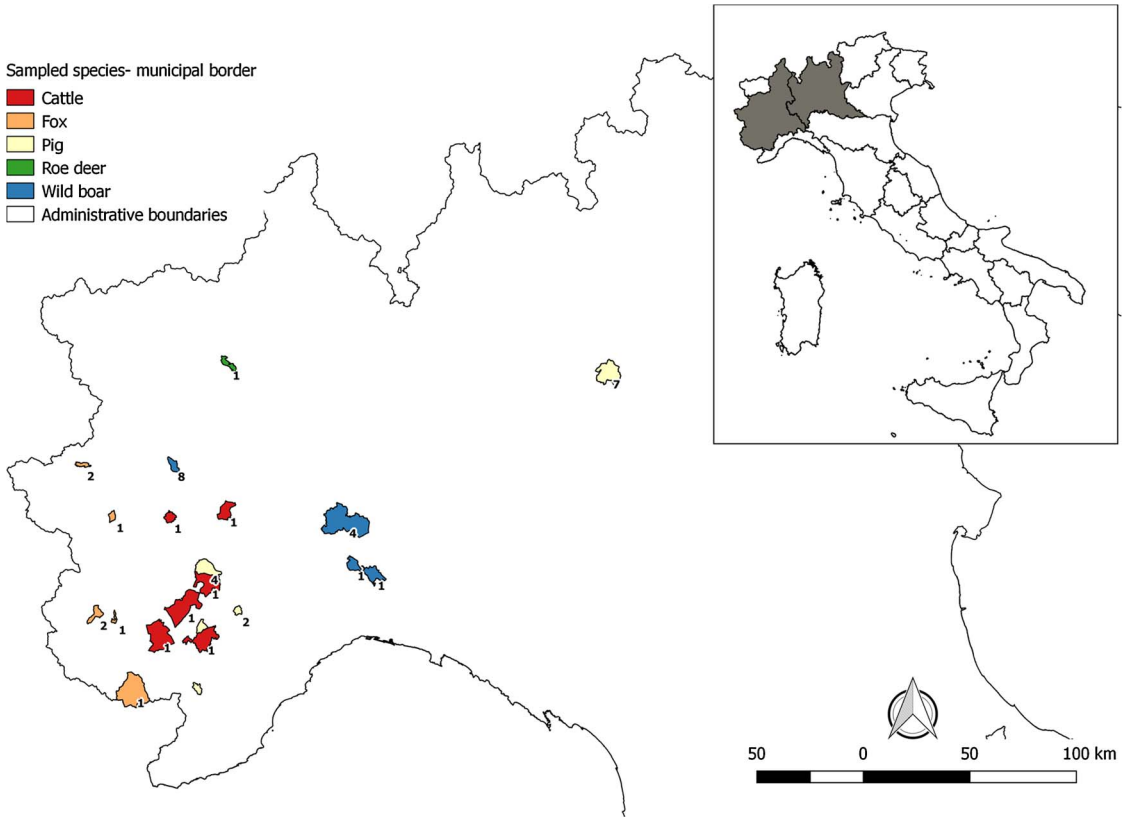

Fig. 1. Sampling sites for both wildlife and livestock. Coloured areas display the municipal borders in which samples were collected; for each area, the number of collected animal samples is shown.