***Toxoplasma gondii* seroprevalence in beef cattle raised in Italy: a multicenter study.**

Alessia Libera Gazzonis[1*], Anna Maria Fausta Marino[2], Giovanni Garippa[3], Luca Rossi[4], Walter Mignone[5], Valter Dini[6]; Renato Paolo Giunta[2], Mario Luini[7], Luca Villa[1], Sergio Aurelio Zanzani[1], Maria Teresa Manfredi[1]

[1] Department of Veterinary Medicine, Università degli Studi di Milano, Milan, Italy

[2] Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", Centro di Referenza Nazionale per la Toxoplasmosi (Ce.Tox.), Palermo, Italy

[3] Department of Veterinary Medicine, University of Sassari, Sassari, Italy

[4] Department of Veterinary Science, University of Turin, Turin, Italy

[5] Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Imperia Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta

[6] Health Veterinary Service, A.S.L. Savonese 2, Savona, Italy.

[7] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Lodi, Italy

* AL Gazzonis, corresponding Author: alessia.gazzonis@unimi.it

Abstract

Toxoplasmosis represents an important public health issue, with the consumption of raw or undercooked meat being a major way of human infection. The role of beef in the transmission of the parasite to humans is questioned due to lower quantity of tissue cysts compared to other meat-producing species. However, the habit of consuming raw beef is regionally diffused, and the risk posed by *T. gondii* infection in cattle should not be overlooked. Therefore, to update information on *T. gondii* in cattle reared in Italy, a multicentric seroepidemiological survey was designed and implemented in four Northern regions (Liguria, Lombardy, Piedmont, and Trentino Alto Adige) and Sardinia.

Overall, a convenience sampling was performed, collecting 1,444 serum samples from 57 beef cattle herds. Thirteen beef breeds were sampled, besides cross-breed; bovines age varied from three months to over 12 years. Sera were tested with

a commercial ELISA for the detection of anti-*T. gondii* antibodies. Individual and data on history movements of animals were analyzed by a generalized linear model.

A *T. gondii* seroprevalence of 10.2% was recorded, with differences among regions and values ranging from 5.3% in Liguria to 18.6% in Piedmont region (*p*-value=0.0001). Both young and adult animals and males and females tested positive, without any significant difference (age and gender: p-value>0.05). Lower seroprevalence values were recorded in cattle born in Italy (8.7%) if compared to animals imported from abroad (13.4%) (*p*-value=0.046). The spread of *T. gondii* in beef cattle destined to Italian consumers is confirmed, suggesting the need of continuous monitoring of the infection.

Key words Serology, food-borne parasite, toxoplasmosis, zoonoses, beef cattle

Introduction

Toxoplasmosis is a zoonotic parasitic disease with a worldwide distribution. Though usually asymptomatic, toxoplasmosis can seriously impact on the health of immunocompromised patients or if acquired during pregnancy (Weiss and Dubey, 2009). Furthermore, chronic infection can be reactivated following immunosuppression due to organ transplants or HIV infection (Weiss and Dubey, 2009).

In humans, a part the vertical transmission (congenital infection, from mother to fetus), the horizontal transmission may occur through the ingestion of oocysts from environmental contamination of water, soil or food and, in less extent, through the ingestion of tissue cysts of infected intermediate hosts (Pinto-Ferreira et al., 2019).

Food producing animals have a different significance in the transmission of *T. gondii* to humans, due to species-related differences in prevalence and intensity of tissue cysts in the edible parts (Stelzer et al., 2019). Compared to other species (i.e., game, sheep, goats, pigs), cattle are deemed to play a limited role in the transmission of *T. gondii* to humans (Guo et al., 2015).

However, number of surveys have shown that the risk for humans is also function of the consumers' eating habits, which largely vary between regions or countries due to different culinary traditions (Guo et al., 2015; Belluco et al., 2018). In Italy, the annual per person consumption of beef has increased slightly in recent years (17.2 kg in 2018, +2% compared to the previous year), although lower than that of pork (38.2 kg), probably also due to the higher cost of beef (10.86-11.59 €/kg, depending on the cuts) compared to pork (7.39 €/kg) (http://www.ismeamercati.it). The consumption of raw bovine meat is part of the cultural and traditional heritage in many countries and also characterizes the new food trends. In Italy, consumers' preferences set beef at putatively high risk of transmitting the infection, as beef is more often consumed raw or undercooked compared to other animal species (Belluco et al., 2018). Recently, the European Authority for Food Safety (EFSA) reconsidered the hierarchical scale of the risk deriving from the consumption of raw or undercooked meat and stated that beef represents a health risk more than what was believed in the past (EFSA, 2018).

Hence, a large-scale sero-epidemiological serosurvey was planned, targeting beef cattle raised in Italy, with the aim of obtaining information on their exposure to *T. gondii* infection in two study areas, Northern Italy and Sardinia Island, which differ geographically and for cultural and food habits.

In fact, the consumption of beef meat is higher in northern regions (58.6% of the population consume beef several times a week) compared to Sardinia (53.3%) (http://dati.istat.it/Index.aspx?QueryId=16813). In addition, traditional cuisine includes various raw beef preparations in many regions of northern Italy: tartare, carpaccio, and beef cured meat, some of which with Protected Geographical Indication (e.g., Bresaola, carne salada, slinzega, mocetta).

Since the correlation between the serological data and the presence of cysts in the edible parts in cattle is poor (Opsteegh et al., 2019), the detection of antibodies does not reflect the infectivity of the meat but gives an indication

of the population exposure to the infection . In parallel, risk factors were analyzed with a special focus on origin and movement of the investigated cattle.

Material and methods

Ethics statement

No animals were sampled for the purposes of the present study; aliquots from serum samples previously taken for unrelated national surveillance program were used. Serum samples and farms were coded, and serological and data analyses were performed blinded.

Sample and data collection

A convenience sampling was carried out including beef cattle from farms located in two separate Italian macro-areas: mainland northern Italy (Lombardy, Piedmont, Liguria and Trentino Alto Adige regions) and insular Italy (Sardinia Island), representative of a high variety of management systems and of differences in landscape and climate. Overall, 1,444 serum samples (Lombardy: 267, 8 farms; Piedmont: 226, 8 farms; Liguria: 434, 19 farms; Trentino Alto Adige: 157, 7 farms; Sardinia: 360, 15 farms) were selected among those collected between October 2016 and May 2017 by local veterinarians in conjunction with routine sampling for regional sanitary controls. For farms with fewer than 50 animals, all animals were included in the study; for farms with more than 50 animals, a representative animal sample of approximately 10% (10-18%, average 12%) of the herd was included in the study. A mean of 24,9 animals was sampled per farms. Serum samples were stored at -20°C until analyzed.

Both young and adult animals were included in the survey, with age varying from three months to over 12 years (mean in months ± standard deviation: 63.2±53.2). Besides cross-breed, both purely beef breeds (Piedmontese, Sardo-Bruna, Podolica, Charolaise, Limousine, Aubrac, Blonde D'Aquitaine, Gasconne) and dual-purpose breeds (Alpine Grey, Alpine Brown, Pezzata Rossa Italiana, Salers, and Valdostana Pezzata Rossa) were sampled.

Individual data (gender, age, and breed) were collected. GPS (Global Positioning System) coordinates of each farm were gathered to map its location. Furthermore, altitude of municipalities where the farms were located was recorded (https://www.istat.it/it/archivio/156224).

Serological analysis

Sera were analyzed with a commercial ELISA (ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET, Montpellier, France) for the detection of anti-*T. gondii* antibodies, performed according to the manufacturer's instruction. Positive and negative control sera provided with the kit were used as controls. For each sample, the resulting values were calculated

by applying the formula supplied in the kit: S/P%= OD sample − OD-negative control/OD-positive control – OD-negative control) × 100. Samples with SP% ≥ 50% were considered positive.

Statistical analysis

The seroprevalence at individual level was computed with the associated 95% confidence interval. A herd was considered positive if at least one seropositive animal was found. For descriptive statistics, age and altitude, respectively computed in months and meters above sea level (m a.s.l.), were categorized.

Univariate binary logistic regression analysis was performed to determine factors that could be considered predictors of seropositivity to *T. gondii* (dichotomous animal-level outcome: seropositive or seronegative). Both individual and herd data were considered: gender, age (continuous variable), breed, breed purpose, region. The Wald's test was used to assess the association of significant variable to *T. gondii* seropositivity. Subsequently, all the variables showing a *p*-value <0.1 and their two-way interactions were entered in a multivariate model, developed by backward elimination until all remaining variables were significant (*p*-value<0.05). Confounding was evaluated as >20% change in the odds ratio. Given a possible clustering of the serological status of animals from the same farm, the variable "farm" was entered as a random factor. Statistical analysis was performed with SPSS (version 19.0; SPSS, Chicago, IL, USA).

Results

T. gondii seroprevalence was 10.2% (95%CI: 8.79-11.92), with 148 seropositive animals out of 1,444 tested. S/P% values of positive samples ranged from 50.0 to 266.1 (mean ± standard deviation: 87.8±37.3). The majority of the herds (39/57, 68.4%, 95% CI 55.52-78.99) had at least one *T. gondii* seropositive animal. The proportion of seropositive cattle tested within each herd ranged between 0% and 42.9%, being ≥ 30% in four farms. Both young and adult animals tested positive and similar prevalence values were obtained in males (12.3%) and females (10%). Particularly, an increase of seroprevalence was evidenced in females until the age of 3 years, whereas males scored positive only until 2 years of age. A higher number of sero-reactors were detected below 200 and between 200-400 m a.s.l. (34/278, 12.2% and 36/233, 15.5%), compared to those from municipalities located at 400-600 and >600 m a.s.l. (28/405, 6.9% and 13/160, 8.1%). Descriptive statistics and results from statistical analysis are reported in Table 1.

Statistical analysis performed by univariate logistic regression analysis showed that the origin of the animals was associated with a higher infection risk. In particular, the seropositivity values differed significantly according to region and animals imported from abroad were at higher risk of infection than cattle from Italy. Seropositivity was also associated to purpose. Any of the variables and of their two-way interactions entered in the multivariate model were not significantly associated to *T. gondii* infection, not even when the variable "farm" was included as random factor.

Discussion

The present study is evidence that *T. gondii* infection is widespread among beef cattle in Italy, with 10.2% of the samples testing seropositive and 68.4% of seropositive farms. The exposure to the pathogen is thus confirmed, as suggested by previous serosurveys conducted in the same study area in other domestic animals (Gazzonis et al., 2015, 2018a, 2020; Villa et al., 2018) and wildlife (Zanzani et al., 2016; Gazzonis et al., 2018b,c). The obtained results are in line with other similar surveys in Europe, in which seroprevalence values ranged between 7.8 % up to 76.3% (Klun et al., 2006; Gilot-Fromont et al., 2009; Berger-Schoch et al., 2011; Jokelainen et al., 2017; Blaga et al., 2019).

A significant difference was observed between dual-purpose breeds and purely beef breeds or crossbreeds, probably for differences in breeding systems, as reported also for other species including goats (Gazzonis et al., 2015): breeds with higher production are usually raised with more intensive systems, while more rustic breeds can be adapted to more mountainous territories and extensively bred. Moreover, a possible influence of genetic differences existing among breed on the susceptibility or in the antibody response to *T. gondii* should be taken into account (Jokelainen et al., 2017).

Age was not associated with differences in seropositivity between young and adult animals, as previously reported (Gilot-Fromont et al., 2009) but in contrast to other studies (Berger-Schoch et al., 2011; Blaga et al., 2019). Interestingly, in our study female and male animals differed in age-related seroprevalence values. In males, anti-*T. gondii* antibodies were only found in animals younger than 2 years, while in females the percentage of seropositive animals increased with age up to three years and a clear pattern was no longer evident in older animals. Possibly, unlike females, most males under 2 years of age includes the category of young animals imported from abroad, extensively reared until being imported in Italy and thus highly exposed to the risk of acquire *T. gondii* infection at pasture. In a study by Jokelainen et al. (2017), a trend to higher seropositivity values was found up to 5 years of age, while a higher variability in seroprevalence values was pictured in older animals.

The lack of age differences is a further evidence that anti-*Toxoplasma* antibody response is not lifelong in cattle: according to Opsteegh et al. (2011), anti-*Toxoplasma* antibodies persist shorter than lifelong in cattle, implying a mismatch between the serological data and the presence of tissue cysts. In cattle, seronegativity does not rule out the presence of the parasite DNA in tissues including meat and other edible organs (Opsteegh et al., 2019). On the other hand, seropositive cattle indicate that cattle are exposed to the risk of infection, and that consumers will be potentially at risk by eating their meat and derived products.

The origin of *T. gondii* infection in humans is often unknown. The way of infection through the ingestion of oocysts in the environment, water or contaminated food seems to be more efficient than the way of infection through the

consumption of meat containing tissue cysts (Pinto-Ferreira et al., 2019). Nevertheless, undercooked beef is considered a source of *T. gondii* for humans, especially in countries and regions where beef is often eaten raw, cured or under-cooked (EFSA and ECDC, 2018). The thermal treatments the meat is subjected to during cooking (at temperatures above 60°C) or freezing (at -20°C for 3 days) are instead able to inactivate the tissue cysts, as demonstrated by several experimental studies recently reviewed (Alizadeh et al., 2018). Considering the Italian settings, and particularly in Piedmont many traditional dishes are based on raw, such as tartare and carpaccio, and beef cured meat. Furthermore, in Italy the sale of fresh beef is far higher (817305 tons sold) than the sale of frozen beef (15065 and 38128 tons sold of Italian and imported frozen meat, respectively). In particular, the import of live animals destined for slaughter (about 79 thousand animals imported in 2019) mainly takes place from countries belonging to the European Union (especially Poland, France and the Netherlands). Only to a lesser extent does import occur from non-European countries (e.g., Argentina) (http: // www. ismeamercati.it), which could represent a risk due to the possible presence of atypical genotypes of *T. gondii*, potentially more virulent than those present in Europe (Shwab et al., 2014).

The serosurvey performed in Italy demonstrated the largest number of seroreactors in Piedmont. It should be noted that in the present study the prevalence was calculated at the individual level, and therefore the data interpretations at the herd level should be taken with caution. A possible explanation could be linked to the breeding system in this region: farms are often extensive, small family-run farms, with the possibility for animals to graze, thus contributing to the completion of *T. gondii* cycle, as previously highlighted (Klun et al., 2006; Gilot-Fromont et al., 2009). Another possible reason for this result lies in the geographical and environmental characteristics of Piedmont, a region with a strong hilly and mountainous component. The altitude was found to be strongly associated to *T. gondii* seropositivity, with the greatest number of positive samples was found at altitudes up to 400 m a.s.l., where the climate conditions (humid and temperate) are more favorable for the survival and sporulation of *T. gondii* oocysts in the environment (Dubey, 2010), as previously demonstrated (Alvarado-Esquivel et al., 2013; Gazzonis et al., 2015).

The same regional differences were reported in *T. gondii* human infection. The incidence rates of hospitalizations due to toxoplasmosis are higher in the northern provinces (Graziani et al., 2016), with 34 notified cases of toxoplasmosis in Piedmont from 2010 to 2018 (https://www.seremi.it). Considering the data obtained from serological screening in pregnant women, the highest seroprevalence value (35.3%) was reported in Piedmont (https://www.ceirsa.org/).

Conclusions

Although seropositivity does not necessarily correspond to the infectivity of the meat and in general of the edible parts, the seroepidemiological data obtained from the present study confirmed the wide diffusion of *T. gondii* in the study area

among beef cattle destined to human consumption. However, regulation at both national and European level concerning the control of *T. gondii* infection both in terms of animal handling and of inspection at slaughterhouses is still lacking today. Therefore, it is of fundamental importance the application of hygienic-sanitary measures at farm level for the achievement and maintenance of an adequate biosecurity to contrast the spread of the pathogen in the beef cattle breeding sector. In parallel, the risk for human infection can be reduced through information and education of consumers on the correct procedures of meat preparation and handling, with a special focus on the appropriate heat treatments (both freezing or cooking) of beef.

Author contributions

ALG, GG, LR, WM, VD, ML conducted the sampling and data collection. AMFM and RPG carried out the serological analysis. ALG carried out the data analysis. ALG, AMFM, GG, LR and MTM wrote the paper. WM, VD, ML, LV, SAZ discussed and commented the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 – Serological data related to *Toxoplasma gondii* in beef cattle from Italy: descriptive statistics, and results obtained in the univariate regression analysis.

Variable category	Seropositive/examined	P% (95% CI)	β±s.e.	Wald's Chi-square	OR (95% CI)	p-value	AIC
Region				30.102		**0.0001**	35.372
Lombardy	24/267	8.9 (6.12-13.03)	-0.838±0.2739		0.433 (0.253-0.740)	**0.002**	
Liguria	23/434	5.3 (3.56-7.83)	-1.406±0.2741		0.245 (0.143-0.420)	**0.0001**	
Sardinia	36/360	10 (7.31-13.53)	-0.720±0.2452		0.473 (0.301-0.787)	**0.003**	
Trentino Alto Adige	23/157	14.6 (9.96-21.02)	-0.285+0.2832		0.752 (0.432-1.310)	0.314	
Piedmont (reference)	42/226	18.6 (14.05-24.16)	0		1		
Age				0.105		>0.1	
≤12 months	35/297	11.8 (8.59-15.94)					
13-36 months	43/334	12.9 (9.7-16.89)					
37-60 months	7/175	4 (1.95-8.03)					
61-84 months	16/218	7.3 (4.57-11.59)					
>84 months	48/419	11.5 (8.75-14.87)					
Gender				1.152		>0.1	
F	121/1224	9.9 (8.34-11.69)					
M	27/220	12.3 (8.57-17.26)					
Breed				16.779		>0.1	
Alpine Brown	0/1	0 (0-79.35)					
Alpine Grey	1/12	8.3 (1.49-35.38)					
Aubrac	1/18	5.6 (0.99-25.76)					
Blonde D'Aquitaine	0/3	0 (0-56.15)					
Charolaise	13/126	10.3 (6.13-16.86)					
Gasconne	0/3	0 (0-56.15)					
Limousine	22/167	13.2 (8.86-19.14)					
Pezzata Rossa Italiana	7/18	38.9 (20.31-61.38)					
Piemontese	50/558	9 (6.86-11.62)					
Podolica	0/1	0 (0-79.35)					
Salers	2/15	13.3 (3.73-37.88)					
Sardo-Bruna	10/74	13.5 (7.51-23.12)					
Valdostana Pezzata Rossa	1/8	12.5 (2.24-47.09)					
Crossbreed	41/440	9.3 (6.94-12.4)					
Purpose				6.109			21.779
Beef	96/950	10.1 (8.35-12.19)	0.822-0.3546		2.276 (1.136-4.560)	**0.02**	
Dual-purpose	11/54	20.4 (11.77-32.9)	0		1		
Origin				3.996			14.743
Imported from abroad	42/314	13.4 (10.05-17.59)	0.485±0.2424		1.624 (1.009-2.611)	**0.046**	
Born in Italy (reference)	35/403	8.7 (6.31-11.83)	0		1		