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Doctoral Thesis

EPIDEMIOLOGY OF SELECTED PROTOZOAN INFECTIONS IN DOMESTIC RUMINANTS.

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General introduction

The interest in food health and safety has grown exponentially over the last years, not only as a consequence of several cases of diseases linked to food (i.e. dioxin contamination or bovine spongiform encephalopathy) but also because consumers are now more attentive to food quality, animal welfare and status health.

Foodborne diseases have therefore received a great attention by public opinion and research institutions. Research mostly focused on pathogens that threaten both food safety and animal welfare. Some of these infections are well recognized; others are considered emerging or re-emerging since their spread is growing and/or they are being more detected thanks to the improving of diagnostic tools and media communication.

Foodborne infections are mostly associated to bacterial infections, such as *Salmonella* serotype Enteritidis, *Escherichia coli* serotype O157:H7, *Campylobacter*, *Listeria monocytogenes*, *Mycobacterium bovis* (Dorny et al. 2009). On the contrary, infections by parasites have been for a long time neglected, although certain zoonotic parasites (i.e. *Taenia* spp., *Trichinella* spp., *Sarcocystis* spp. but also emerging or re-emerging parasite such as *Toxoplasma gondii*, *Trypanosoma cruzi* and *Echinococcus* spp.) still represent worldwide a public health problem (Dorny et al. 2009).

Particularly, zoonotic protozoa such as *Cryptosporidium parvum* spp., *Cyclospora cayetanensis*, *Giardia duodenalis*, and *T. gondii*. received increasing attention in the last years since they all have been recognized as waterborne and foodborne pathogens potentially causing important diseases (Dawson 2005).

Focusing on food-borne pathogens, *T. gondii* deceives a major attention. Toxoplasmosis burden is indeed similar to those of other foodborne diseases such as salmonellosis or campylobacteriosis, although toxoplasmosis has received little attention from policy makers in years (Kijlstra and Jongert 2008). With the European legislation on food security (‘Hygiene package’, Regulation (EC) No 852, 853, 854/2004), the European Union established a community framework for official controls on products of animal origin intended for human consumption and laid down specific rules for fresh meat, bivalve mollusks, milk and dairy products. Toxoplasmosis does not figure within diseases subjected to research, in spite of EFSA recommendations that classified *T. gondii* as a high priority for meat inspection, particularly for sheep and goat products (EFSA 2013).

European representative data on *T. gondii* infection in humans, animals or food products are not available and comparable, because of a lack standardization and harmonization within Member

States. Nevertheless, the infection is a major public health concern since it affects one-third of human population (Cook et al. 2000). Though usually asymptomatic in immunocompetent patients, toxoplasmosis can cause encephalitis, pneumonia and myocarditis in immunocompromised hosts affected by chronic infection due to a reactivation of *T. gondii* (Ferreira and Borges 2002, Meroni et al. 2014). Moreover, it causes abortion and congenital infections in approximately 0.1-1‰ of newborns in Europe that may suffer from severe ocular diseases (4–27%), general health problems (1–2%) or even die (Cook et al. 2000).

The consequences of infection by *T. gondii* depend not only on the host (weakness or immaturity of the immune response, susceptibility or resistance factors) but also on parasite characteristics (strain, inoculum size, parasitic stage) (Maubon et al. 2008). In Europe and North America, mainly three *T. gondii* clonal lineages (named types I, II and III) dominate, defined by multi-locus enzyme electrophoresis (MLEE), polymerase chain reaction restriction fragment length polymorphism (PCRRFLP) or microsatellite analysis (Su et al. 2010). In mice, type I lineages are uniformly lethal (LD100=1); by contrast, the type II and III lineages are significantly less virulent (LD100>=103). In humans, disease manifestations vary widely, ranging from asymptomatic to severe acute toxoplasmosis. Type II is the predominant lineage causing human toxoplasmosis. However, there are biases between disease presentations and parasite genotypes. For examples, type I or type I-like atypical isolates are more likely to be involved in severe retinochoroiditis in human patients and the atypical isolates often cause severe acute, disseminated toxoplasmosis in immunocompetent patients (Ajzenberg et al. 2002, EFSA 2007, Su et al. 2010, Dubey et al. 2011).

Multiple routes of transmission are possible in human hosts: by food or water contaminated with oocysts dispersed by cats and other felines (definitive hosts), by consumption of raw or undercooked meat containing tissue cysts or un-pasteurized milk containing tachyzoites, and transplacental transmission. For humans, the consumption of raw or undercooked meat is considered to be one of the most important sources of infection for humans (Cook et al. 2000). However, cysts distribution and number in the meat of intermediate hosts vary among species: tissue cysts are most frequently observed in sheep, goats and pigs, and less frequently in infected poultry, rabbits and horses. Cattle do not seem to represent an important source of human infection although seroprevalence may be very high (Dubey et al. 2005, Kijlstra and Jongert 2008).

In particular, a large number of cysts were demonstrated in meat or cured meat products from ovine or caprine hosts, these species representing a significant way of infection mainly in those regions or countries where mutton and goat meat is routinely prepared (Cook et al. 2000, Kijlstra and Jongert 2008). An additional way of transmission through the consumption of unpasteurized goat or sheep

dairy products was suggested (Cook et al. 2000, EFSA 2007). *T. gondii* DNA was indeed demonstrated in caprine raw milk (Fusco et al. 2007, Mancianti et al. 2013).

Besides important data on humans, also from a veterinarian and zootechnical point of views toxoplasmosis represents a huge problem. Particularly in small ruminants, *T. gondii* is recognized as one of major cause of infectious reproductive failure in several countries of the world causing fetal reabsorption, abortion at any stage of pregnancy, fetal mummification, stillbirth or birth of alive but weak offspring (Ortega-Mora et al. 2007, Rinaldi and Scala 2008, Dubey et al. 2011).

Further, neosporosis is to be considered among the major cause of reproductive failure in small ruminants (Ortega-Mora et al. 2007). Widely spread among domestic ruminants, *Neospora caninum* shares with *T. gondii* a quite similar life cycle, both presenting a carnivore as definitive host: a feline in the case of *T. gondii* and a canine in the case of *N. caninum* (Dubey and Lindsay 2006, Dubey 2010).

Although cases of abortion in small ruminants remain often undiagnosed, probably leading to an underestimation of the diseases, *N. caninum* DNA was found in the brain of caprine and ovine aborted fetuses in different countries (Masala et al. 2007, Howe et al. 2008, Abo-Shehada and Abu-Halaweh 2010, Bishop et al. 2010, Unzaga et al. 2014). A recent study showed very high anti-*N. caninum* antibody levels in the ewes reflecting the existence of an active infection that may cause reproductive failure as the decrease both in the number of births and the rate of viable lambs per ewe (Moreno et al. 2012). Further, *N. caninum* DNA was detected in the brains of 13 out of 14 offspring from seropositive sheep and it was demonstrate that infection by *N. caninum* was the cause of the low reproductive performance of a sheep flock (Gonzalez-Warleta et al. 2014).

From an epidemiological point of view, in Europe a wide range of seroprevalence values for toxoplasmosis were reported (Halos et al. 2010, Opsteegh et al. 2010, Berger-Schoch et al. 2011, Iovu et al. 2012, Tzanidakis et al. 2012, Garcia-Bocanegra et al. 2013, Lopes et al. 2013). Particularly in Italy, values varied from 28.4% to 78% in sheep and from 11.7% to 60.6% in goats (Cenci-Goga et al. 2013, Mancianti et al. 2013). In northern Italy, only few data on infection of small ruminants are available (Gaffuri et al. 2006).

Epidemiological studies on *N. caninum* infection in sheep and goats are scarce and prevalence highly varies depending on the tests and on the sampling area (Dubey and Schares 2011). In Europe, seroprevalence values of 1.9% and 16.8% in sheep and between 2.3 and 9% in goats were registered (Spilovska et al. 2009, Czopowicz et al. 2011, Bartova and Sedlak 2012, Iovu et al. 2012, Diakou et al. 2014); in Italy, high seropositivity were recorded in sheep (66.9%) in Sardinia (Mula et al. 2012), whereas low prevalence (2%) were reported in sheep in Lombardy (Gaffuri et al. 2006).

Another important concern represented by a parasitic disease considered emerging in Europe is bovine besnoitiosis (EFSA 2010). Closely related to *Toxoplasma gondii* and *Neospora caninum*, *Besnoitia besnoiti* is a protozoan parasite belonging to the group of cyst-forming coccidians (Apicomplexa, Sarcocystidae). Similarly to other species belonging to the genus *Besnoitia* infecting ungulates, the life cycle of *B. besnoiti* is in part unknown: cattle represent the intermediate host, whereas the definitive host, if any, has not been yet identified. By analogy with other Apicomplexa species, a carnivore, eventually the cat has been suggested as the definitive host (Basso et al. 2011, Olias et al. 2011). Hematophagous insects (*Glossina*, *Stomoxys* and Tabanids) are considered mechanical vectors (Lienard et al. 2013). Moreover, the possibility of infection through the close contact between animals and iatrogenically (with the repeated use of hypodermic needles), have been suggested as potential means of transmission (Basso et al. 2011, Alvarez-Garcia et al. 2013a). Animal trade and movement throughout countries has been identified as major risk factors for establishment of new bovine besnoitiosis foci in naive areas and countries (Alvarez-Garcia et al. 2013a). Furthermore, the role of wild animals as possible hosts of the parasite needs to be investigated; hitherto, only few cases of seropositivity in red deer and roe deer have been registered in Europe and any surveyed wild carnivores showed antibody against *B. besnoiti* (Millan et al. 2012, Gutierrez-Exposito et al. 2013). Native of Sub-Saharan Africa, in Europe Bovine besnoitiosis is a disease in expansion in Europe both in number of cases of infection and in the distribution of the pathogen. It is endemic in large areas in Spain, Portugal and France, while isolated outbreaks have been reported in Germany, Switzerland, Italy, Greece and Hungary (Agosti et al. 1994, EFSA 2010, Gollnick et al. 2010, Manuali et al. 2011, Mutinelli et al. 2011, Gentile et al. 2012, Alvarez-Garcia et al. 2013a, Alvarez-Garcia et al. 2014, Cortes et al. 2014, Gutierrez-Exposito et al. 2014, Hornok et al. 2014, Papadopoulos et al. 2014, Waap et al. 2014) .

Bovine besnoitiosis is responsible for severe economic losses on affected farms including mortality, weight loss, prolonged convalescence, definitive or transient sterility in males and a decline in milk production, especially in recently affected areas (Cortes et al. 2005, Jacquiet et al. 2010).

According to recent indications of EFSA (2010), epidemiological surveys are necessary to monitor the presence of *B. besnoiti* in Europe and to increase knowledge on biology and associated risk factors.

Aims

The aim of my doctoral project is to investigate on protozoan infections by *Toxoplasma gondii* and *Neospora caninum* in small ruminants and *Besnoitia besnoiti* in cattle. The selected protozoa are nowadays considered (re-)emerging and under-reported in Europe but with important consequences on public health and food security, in addition to animal welfare and health. Different research lines were therefore developed:

Research line 1 – *Toxoplasma gondii* and *Neospora caninum* infections in small ruminants

Epidemiological data on *T. gondii* and *N. caninum* infections in small ruminants bred in Northern Italy were generated; individual and managerial risk factors associated to the infections were analyzed. Moreover, spatial analysis was carried out in order to determine geographical features of Northern Italy able to facilitate or not the maintenance of the infections within the study area.

Research line 2 – Diagnosis of *Toxoplasma gondii* infection in small ruminants by milk and sanitary risks from ovine and caprine products

Following EFSA guidelines (2010), a research line on the investigation of *Toxoplasma gondii* infection in foodstuff was planned. Firstly, a commercial ELISA was validated to analyze goats' milk sample. This allowed carry out analysis in a "case study" caprine farm, previously serologically tested for toxoplasmosis; variation in antibodies response during lactation was observed and analyzed. Finally, two epidemiological surveys were carried out on ovine and caprine products through the detection of antibodies anti-*T. gondii* in bulk tank milk and in meat juice obtained by slaughtered goats and sheep.

Research line 3 – Bovine besnoitiosis in cattle

An epidemiological survey on bovine besnoitiosis was performed on dairy and beef cattle bred in different areas of Italy in which no updated data were available: northern regions (Lombardy and Piedmont), Liguria region and Sardinia Island.

Subsequently, in a seropositive farm an in-depth analysis was performed, applying different diagnostic tools (serology, histology and immunohistochemistry) to better explain the disease in a infected farm.

Research line 1 – *Toxoplasma gondii* and *Neospora caninum* infections in small ruminants

1. Epidemiological survey of *T. gondii* and *N. caninum* infections in small ruminants bred in Northern Italy

1.1. Introduction

Toxoplasma gondii and *Neospora caninum* are two closely related Apicomplexa parasites, presenting worldwide distribution. Both protozoa potentially infect a wide variety of warm-blooded vertebrates, including mammals, birds and humans. Life cycle is similar, presenting a definitive host represented by a carnivore: a feline in the case of *T. gondii* and a canine in the case of *N. caninum* (Dubey and Lindsay 2006, Dubey 2010).

The European Food Safety Authority (EFSA) indicated toxoplasmosis as one of the most important parasitic zoonoses due to its high human incidence and published a recent scientific opinion clearly stating the need for investigation on its occurrence both in humans and animals in Europe (EFSA 2007). Epidemiological data on *T. gondii* infections in animals for human consumption are not regularly collected and current lack of standardization of diagnostic techniques and protocols should be taken into account when comparing seroprevalence data (Tenter et al. 2000).

From a zootechnical point of view, toxoplasmosis and neosporosis are widely spread among ruminants, being among the major causes of reproductive failure. Although cases of abortion remain often undiagnosed, probably leading to an underestimation of the diseases, toxoplasmosis and neosporosis has been reported as a cause of abortion both in sheep and goats (Dubey and Lindsay 2006, Masala et al. 2007, Howe et al. 2008, Rinaldi and Scala 2008, Dubey 2009, Abo-Shehada and Abu-Halaweh 2010, Bishop et al. 2010, Moreno et al. 2012, Gonzalez-Warleta et al. 2014, Unzaga et al. 2014).

Several serosurveys have been carried out in small ruminants' farms mostly on toxoplasmosis. In Europe a wide range of seroprevalence values for toxoplasmosis were reported: individual seroprevalence varies from 27.8% in the Netherlands in sheep (Opsteegh et al. 2010), 33.6% in sheep and 18.5 in goats in Portugal (Lopes et al. 2013), 89% in sheep in France (Halos et al. 2010), 61.6% in sheep in Switzerland (Berger-Schoch et al. 2011), 52.8% in goats in Romania (Iovu et al. 2012), 48.6% in sheep and 30.7% in goats in Greece (Tzanidakis et al. 2012), 49.3% in sheep and

25.1% in goats in Spain (Garcia-Bocanegra et al. 2013), 33.6% in sheep and 18.5% in goats in Portugal (Lopes et al. 2013).

Particularly in Italy, the lowest values were registered in Sardinia both in goats (12.3%) and sheep (28.4%) (Masala et al. 2003). In other regions, values varied from 28.5% to 78% in sheep and from 11.7% to 60.6% in goats (Cenci-Goga et al. 2013, Mancianti et al. 2013). In northern Italy, only few data on infection of small ruminants are available (Gaffuri et al. 2006) (Table 1).

Table 1- Seroprevalence data on T. gondii recorded in small ruminants from different Italian regions over the last 3 decades, adapted from Rinaldi and Scala (2008).

Species	References	Diagnostic method	Prevalence (%)	No. examined animals	Region
GOATS	De Capraris and Gravino, 1981	MAT	95.0	198	Lazio
	Puccini *et al*., 1983	IFAT	68.9	244	Apulia and Basilicata
	Masala *et al*, 2003	IFAT IgG	12.3	2445	Sardinia
		IFAT IgM	5.6		
	Tola *et al*., 2006	IFAT IgG	11.7	4562	
		IFAT IgM	4.0		
	Mancianti *et al*., 2013	MAT	60.6	127	Tuscany
	Gazzonis *et al*., 2014	IFAT	41.7	474	Lombardy
SHEEP	Gaffuri *et al*., 2006	LAT	78.0	352	Lombardy
	Gazzonis *et al*., 2014	IFAT	59.3	502	Lombardy
	Baldelli and Pietrobelli, 1985	IFAT	69.0	374	Emilia Romagna
	Cenci-Goga	IFAT IgG	33.3	630	Tuscany
	Fusco *et al*., 2007	IFAT	28.5	1170	Campania
	Puccini *et al*., 1981	IFAT	88.6	306	Apulia and Basilicata
	Puccini *et al*., 1983	IFAT	56.1	321	
	Balbo *et al*., 1980	IFAT	0.1	1390	Sicily
	Vesco *et al*., 2007	ELISA	49.9	1876	
	Masala *et al*., 2003	IFAT IgG	28.4	7149	Sardinia
		IFAT IgM	9.9		
	Tola *et al*., 2006	IFAT IgG	19.2	29886	
		IFAT IgM	5.4		
	Natale *et al*., 2006	ELISA	51.3	1043	
	Zedda *et al*.,2009	IFAT IgG	31.5-62.6	422	
		IFAT IgM	14.9		

Legend: DAT = direct agglutination test; IFAT = indirect fluorescent antibody tests; MAT = microscopic agglutination test; LAT = latex agglutination test; ELISA = enzyme-linked immunosorbent assay.

Epidemiological studies on *N. caninum* infection in sheep and goats are scarce and prevalence highly varies depending on the tests and on the sampling area (Dubey and Schares 2011). Higher seroprevalence values were recorded in sheep than in goats by several authors: Nasir et al. (2012a) registered 27.7% of seropositive sheep and 8.6% of goats in Pakistan, similarly Abo-Shehada and Abu-Halaweh (2010) in Jordan 63% and 2% of seropositive sheep and goats, respectively. In Brazil, lower values were recorded in several surveys both in sheep (1.8-9.2%) and goats (3.3-6.4%) (Figliuolo et al. 2004a, Figliuolo et al. 2004b, Soares et al. 2009, Machado et al. 2011,

Topazio et al. 2014). In Europe, seroprevalence values of 1.9% and of 3.7% were reported in sheep in Spain and Slovakia respectively (Spilovska et al. 2009); in goats, Iovu et al. (2012) registered 2.3% and Bartova and Sedlak (2012) 6% of seropositive goats at individual level, while (Czopowicz et al. 2011) 9% of caprine farms at herd level. Diakou et al. (2014) investigated on mixed flock, recording 16.8% and 6.9% individual prevalence in sheep and goats, respectively.

Concerning Italy, a recent seroepidemiological survey revealed high positivity in sheep (66.9%) from 5 sardinian flocks (Mula et al. 2012) and less recently the infection was diagnosed in a goat foetus by PCR and histopathological analysis (Eleni et al. 2004). Gaffuri et al. (2006) reported low prevalence (2%) in sheep in Northern Italy; recent data on both sheep and goats bred in northern Italy however lack.

The present study therefore aimed to update information on the seroprevalence of *T. gondii* and *N. caninum* infection in small ruminants from a region of northern Italy, Lombardy, where these animals, goats in particular, are of relevant importance. Possible risk factors associated with the infection were also considered and evaluated.

1.2. Materials and methods

Study area description. The survey was carried out in northern Italy in some areas of Lombardy (45°40'N, 9°30'E), the most suitable for goat and sheep breeding, i.e. in the southern province of Milan, eastern of Bergamo and western of Varese. They were selected considering their wide sheep and goat population, their varied animal management systems and their different landscape and climate (http://www.scia.sinanet.apat.it/sciaweb/scia_mappe.html). The province of Milan (1575km^2) is mainly flat and the altitude of sampled farms ranges from 80 to 220 ma.s.l. The area has a considerable farming activity mainly characterized by large intensive goat farms focused only on milk production. The territories of Bergamo (2745km^2) and Varese (1199km^2) show flatland (95 and 194 ma.s.l., respectively), hills and mountains of the Lombard Alps (1508 and 896 ma.s.l., respectively). In these provinces farms are smaller and produce traditional cheese directly from milk. In Lombardy, sheep transhumance is still practiced. In the winter, sheep are moved from alpine pastures to Milan's lowlands following the main routes (north to south) passing through Bergamo province down to the River Po plain areas. Goats raised in extensive farms usually graze from March or May to October or November during the day (or at night in the hottest months) and are kept in a fold at night (or during the day in the hottest months), depending on the area.

Study population and sample collection. Data obtained by ISTAT (ISTAT 2010) showed that in the province of Milan (southern area) there were 7153 small ruminants (27 sheep and 47 goat

farms), 49218 (89 sheep and 365 goat farms) in Bergamo (eastern area), and 9238 (109 sheep and 186 goat farms) in Varese (western area). A minimum sample size was determined by using the program Winepiscope 2.0 (http://www.clive.ed.ac.uk/winepiscope/0) to exclude (if all samples are negative) a *T. gondii* or *N. caninum* seroprevalence ≤15% within the animals in the sampled herds at a confidence level (CI) of 95%.

Overall 502 sheep and 474 goat blood samples from 45 farms were collected between October 2012 and May 2013; all samples were available for investigation on *T. gondii*, whereas 428 and 414 sera were processed for the research on neosporosis. Ten farms (4 goat, 3 sheep and 3 mixed farms; mean 120 animals, min-max 20-500) located in the southern area, 22 (17 goat, 4 sheep and 1 mixed farms; mean 27, min-max 10-200) in the western area and 13 (3 sheep and 10 mixed farms; mean 1100, min-max 250-1600) in the eastern area were sampled.

GPS (global positioning system) coordinates of each farm were gathered to map its location. Within each selected flock/herd, animals (aged 4-159 months) were sampled by systematic random selection, proportionally to the total number of adults present in the farm. Regarding sampled goat breeds, two were cosmopolite (Alpine and Saanen), one autochthonous (Nera di Verzasca) and others crossbreed. As to sheep breeds, one was cosmopolite (Merinos), one local (Bergamasca), and others crossbreed. Blood samples were collected from jugular vein and preserved in tubes without anticoagulants. Sera were separated by centrifugation (15 min, 2120 g) and stored at -20 °C until serological testing.

Questionnaire data collection. A questionnaire about farm management was submitted and filled out by farmers and veterinarian practitioners at sampling time. It included questions on rearing system (extensive, intensive or semi-intensive for goats; extensive, semi-intensive or transhumant for sheep), species bred (only sheep or goats or mixed), farm size (number of animals on farm), possibility to graze, nutrition (only grazing or supplementation with feeding concentrate), water source (stagnant water source or municipal water), purchase of spare breeding animals, presence of other species in the farm or sharing grazing (bovine, equine, wild ungulates) or domestic animals in the farm (dogs, swine or poultry), presence of resident and/or stray cats on the property. Besides, individual data on animals (sex, age, breed) and on the location of the farm (province and altitude) were collected.

Serology for *T. gondii*. Serum samples were analyzed using a commercial indirect immunofluorescence antibody assay (IFAT) to determine the presence of IgG antibodies against *T. gondii*. The serological test was performed according to the method described by Camargo (Camargo 1974) using slides spotted with whole RH strain tachyzoites(Mega CorDiagnostik, Horbranz, Osterreich, Austria) as antigens and fluorescein isothiocyanate-labelled rabbit anti-sheep

IgG (whole molecule, Sigma-Aldrich, St Luis, MO, USA) diluted 1:100 in PBS plus 0.01% Evans blue as conjugate for sheep. For goats, fluorescein isothiocyanate-labelled rabbit anti-goats IgG (whole molecule, Sigma-Aldrich, St Luis, MO, USA) diluted1:200 in PBS plus 0.01% Evans blue was used as conjugate. Sera were screened considering 1:64 dilution as the cut-off and those testing positive were serially two-fold diluted to determine the end-point titre(Figliuolo et al. 2004a, Figliuolo et al. 2004b). Positive and negative controls were included in each assay and the slides were examined under a fluorescence microscope (Axioscope 2, Zeiss) at 400 or 1000× magnification. Only a bright, linear, peripheral fluorescence extended to whole the body of the tachyzoites was considered as positive reaction.

Serology for *N. caninum*. Serum samples were analyzed for anti-*Neosporacaninum* antibodies by a in-house ELISA as described by Aguado-Martinez et al. (2008). Sera resulted positive were subsequently analyzed by Western Blot (WB) as a confirmatory test, as suggested by several authors (Alvarez-Garcia et al. 2013b). As control for both tests, bovine positive and negative sera samples previously tested both by IFAT and WB were used (Alvarez-Garcia et al. 2002).

ELISA. Sera were analyzed through a standardized in house ELISA with the soluble antigens prepared from tachyzoites of Nc-1 isolates as target as described by Aguado-Martinez et al. (2008). Briefly, 100 µl of coating buffer (0.1M carbonate-bicarbonate, pH 9.6) containing *N. caninum* soluble extract (0.15 µg) was added to each well of a polystyrene microtitre plate (Immuno Plate Maxisorp; Nunc, Roskilde, Denmark) and incubated overnight at 4ºC, then washed with Phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST) and finally blocked for 2 h at room temperature with PBST containing 3% Bovine Serum Albumin (BSA). Wells were washed three times with PBST and incubated with 100µl of goat or sheep sera diluted 1:100 in blocking solution, for one hour at 37ºC, then washed again. One hundred microliters of antisheep or antigoat monoclonal IgG (Sigma-Aldrich, Madrid, Spain) conjugated with peroxidase diluted 1:5000 in PBS-T plus BSA 3% were added to each well and left for one hour at 37ºC. After three washes with PBST, 100µl of 2,2′-Azino-bis (3-Ethylbenzthiazoline- 6-sulfonic acid) substrate (Sigma-Aldrich, Madrid, Spain) was added and incubated 1 h at room temperature in the dark. After 20 minutes, the reaction was stopped by adding 100µl of 0.3M oxalic acid to the wells. Absorbance was measured as optical density (OD) values at 405nm using a microplate reader (Multiscan RC 6.0; Labsystems). Samples were analyzed in duplicate and the mean value of the OD was converted into a relative index percent (RIPC) by employing the following formula:

RIPC = (OD sample - OD negative control)/(OD positive control - OD negative control) × 100.

For both species, the cut-off value was calculated using a panel of 30 sera resulted negative both to *Toxoplasma gondii* by IFAT and to *N. caninum* by Western Blot. The mean of the optical density

(OD) and the standard deviation (SD) of the OD values were calculated. The cut-off value was finally obtained according to the following formula: cut-off value = OD mean + 3SD. ELISA cut-off OD values were calculated at 0.226 and 0.267 for goat and sheep, respectively. Samples with OD values greater than the cut-off were considered positives.

Western Blot. WB was performed under reducing conditions according to Alvarez-Garcia et al. (2002). Samples containing 2×10^7 *N. caninum* tachyzoites were deterged-disrupted at 95°C for 5 min with Laemmli buffer (Laemmli 1970), sonicated in an ultrasonic bath at 15°C for 15 min and then heated for 5 min at 95°C prior to use. Electrophoresis was performed in 15% polyacrylamide-DATD minigels and then transferred to a nitrocellulose membrane (Mini Trans-Blot Cell, Bio-Rad Laboratories, CA, USA). Precision Plus Kaleidoscope weight standards (Bio-Rad Laboratories, CA, USA) were subjected to electrophoresis to estimate the apparent molecular weights of the different antigens recognized by sera. Membranes were washed in Tris -buffered saline (TBS) with 0.05% Tween-20 (TBS-T), and then incubated overnight in blocking buffer (TBS-T, containing 3% (w/v) bovine seroalbumin) (Roche Molecular, Biochemical, Mannheim, Germany). After washing in TBS-T, membranes were incubated with sampled sera diluted 1/20 in blocking buffer for 1 h at 37°C, washed again and exposed to anti-ovine or anti-caprine monoclonal IgG (1:300) conjugated with peroxidase (LSI laboratories) and incubated for 1 h at 37°C, rewashed and finally developed using 4-chloro-1-naphtol (Bio-Rad Laboratories) as substrate. Images from the membranes were obtained using a GS-800 Scanner (Bio-Rad Laboratories, CA, USA) and analyzed with Quantity One1 quantification software v. 4.0 (Bio-Rad Laboratories, CA, USA). Samples were considered positive if presented immunodominant bands (IDAs) in both area I (17-18 kDa) and area II (34-35 kDa).

Statistical analysis. The seroprevalence at individual and farm level was computed with the associated 95% confidence interval. A farm was considered positive if at least one seropositive animal was found. Pearson's chi-square was used to test for difference between the species. If significant difference would be registered, separate analysis of potential risk factors would be conducted for goats and sheep. Two models of univariate binary logistic regression analysis were performed to determine factors that could be considered predictors of seropositivity to *T. gondii* or *N. caninum*, respectively. All the answers from the questionnaire were included in the statistical analysis as independent variables. Subsequently, all significant variables ($p<0.05$) were entered in a multivariate model which was developed by backward elimination. In the analysis on *N. caninum* infection regarding sheep data, the variable "purchase of spare breeding animals" was not included because any sampled farm bought animals outside. Moreover, data on seropositivity to *T. gondii*

were included in the statistical analysis on *N. caninum* infection as an independent variable. Statistical analysis was performed with SPSS software (version 19.0; SPSS, Chicago, IL).

1.2.1. *Toxoplasma gondii* infection: Results

Antibodies to *T. gondii* were found in 28 (96.6%; 90-100%, 95% CI) out of 29 goat farms, whereas 21 (87.5%; 74.3-100%, 95% CI) out of 24 sheep farms showed at least one seropositive animal. In Figure 1, the spatial distribution of positive farms is represented.

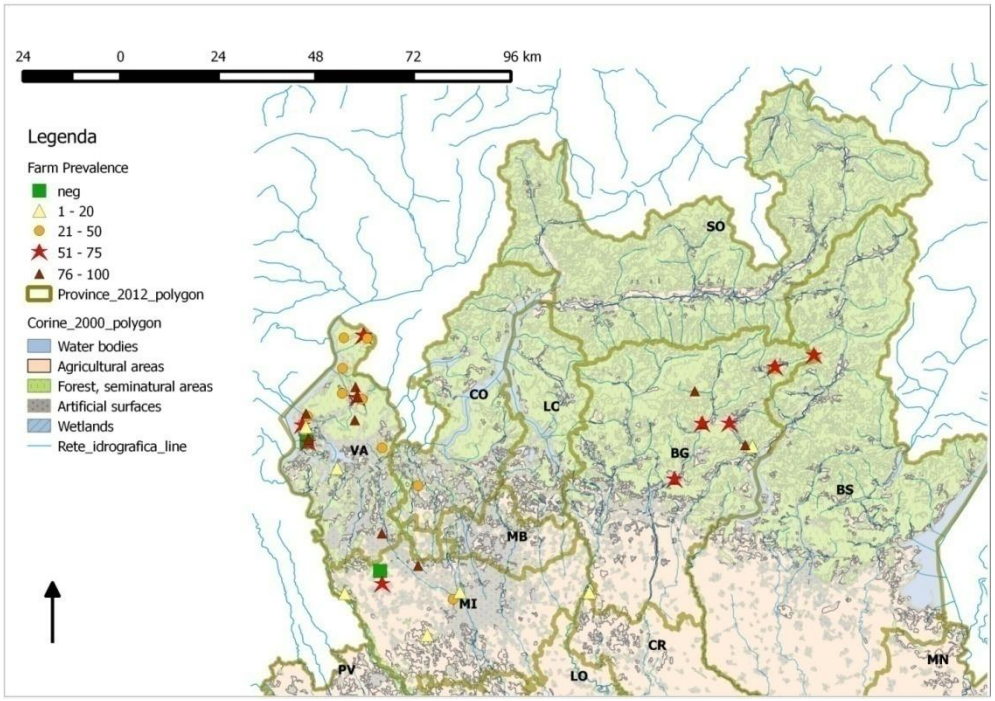

Fig. 1 - Map of location of sampled farms in three surveyed areas of northern Italy (VA= western area, MI= southern area and BG= eastern area). Different markers represent different seroprevalence values.

At individual level, 41.7% (198/474) of goats and 59.3% (298/502) of sheep resulted positive. Most of small ruminants were seropositive with titers of 1:64; higher antibody titres (1:512) were found in 4.2% and 3.6% of goats and sheep, respectively (Table 2).

Table 2 - Rates of infection with *T. gondii* in studied population according to animal species and antibody titer.

	Titre								Total	
	1:64		1:128		1:256		1:512			
	N (%)	95%CI	N (%)	95%CI	N (%)	95%CI	N (%)	95%CI	N (%)	95%CI
Goats (474)	88 (18.6)	15.1-22.1	39 (8.2)	5.7-10.7	54 (11.4)	8.5-14.3	17 (3.6)	1.9-5.3	198 (41.7)	37.2-46.1
Sheep (502)	152 (30.3)	26.3-34.3	50 (10)	7.4-12.6	75 (14.9)	11.8-18	21 (4.2)	2.4-6	298 (59.3)	55.1-63.7

n: number of animals; %: seroprevalence; 95% CI: 95% Confidence Interval

According to host species, the distribution of infected animals varies among farms. As to goats, in 8 farms (27.6%) all tested animals had antibodies to *T. gondii*; in 6 farms (20.7%) more than 60% were seropositive and in 5 farms (17.24%) ≥50% or <60% were seropositive (Fig. 2A). As to sheep, in all farms seronegative animals were found; in 11 farms (45.8%) more than 60% were seropositive and only 1 farm had a percentage ≥50% or <60% of seropositive sheep (Fig. 2B).

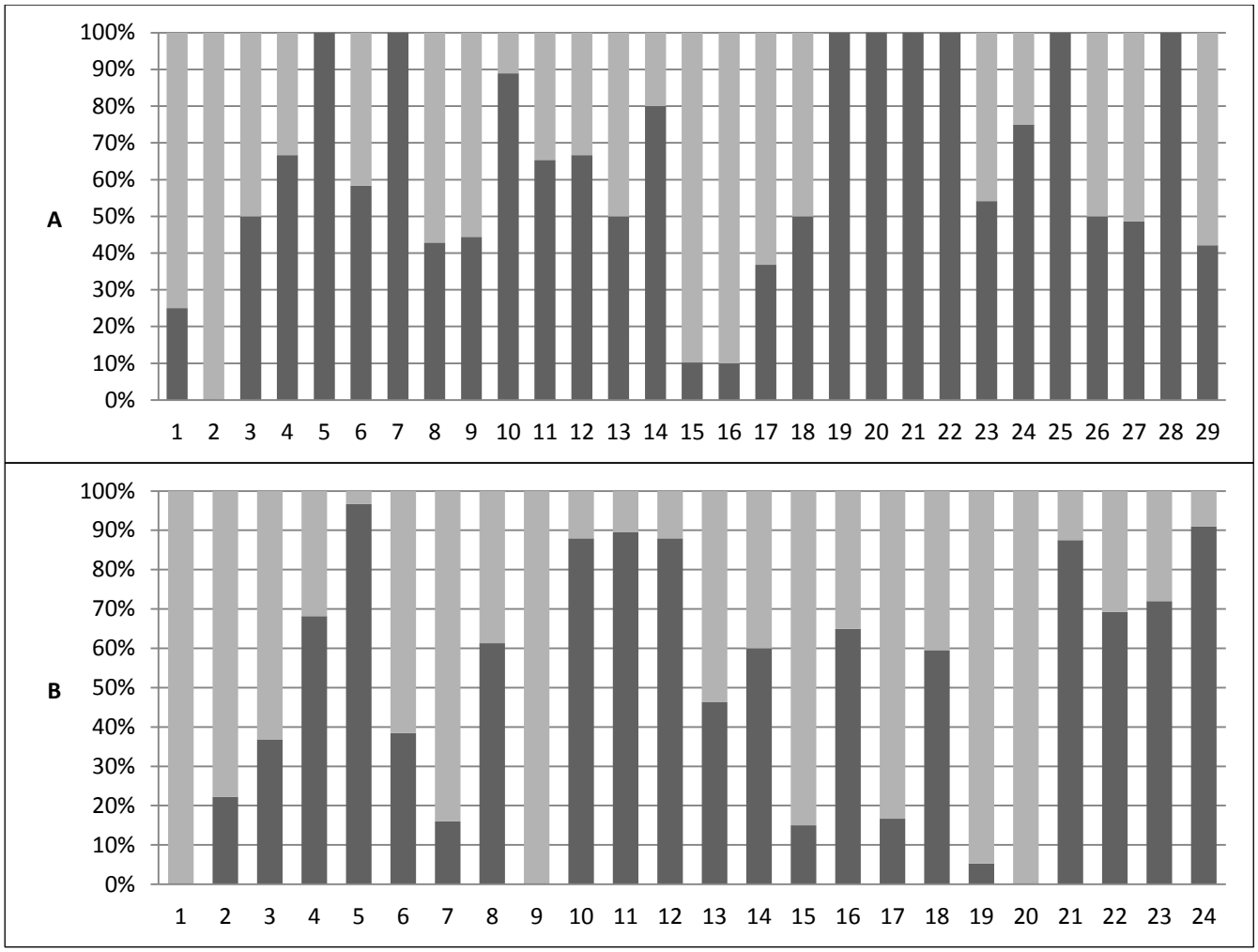

Fig. 2 - Proportion of *T. gondii*-seropositive (black) and negative (grey) animals in 29 goat farms (A) and 24 sheep farms (B) in northern Italy.

According to age, in goats the highest percentage of infection was found in 4 year-old animals, whereas in older animals (> 6 years) the percentage of seropositive animals decreases. In sheep, seroprevalence directly increases with the increasing of age, and the highest seroprevalence values were registered in animals > 6 years old.

Seroprevalence was significantly higher in sheep than in goats (Pearson's chi-square, p=0.0001), therefore analysis of potential risk factors was separately conducted for the two species.

Results obtained from risk factor univariate analysis for goats are given in Table 3. For goats, animals sampled in eastern and western areas showed similar seroprevalence values; goats from

southern area had a lower probability to become infected (Odds ratio (OR) = 0.412; 95% CI: 0.194-0.876). Considering breeds, Saanen goats presented the lowest seroprevalence (30.7%) whereas crossbreeds exhibited the highest level (48.7%). The risk for a Saanen of being infected is 0.554 times smaller than for a Nera di Verzasca (OR= 0.554; 95% CI: 0.319-0.959%). Goat age was one of the strongest predictors of *T. gondii* infection; the odds for a goat of being infected were 1.011 times greater for every 1 month increase in age. The risk factor "number of animals on farm" resulted highly significant; increasing the number of animals on a farm of one unity, their risk to become seropositive was 0.998 lower. Goats from farms breeding both sheep and goats had a risk of being infected 1.396 times higher than goats from farms housing no sheep. Regarding the variable "rearing system", animals bred in intensive farms showed lower prevalence (22.1%) in comparison with those bred in extensive (45.6%) or semi-intensive ones (60%); the risk of infection increases from intensive farms (OR= 0.189; 95% CI: 0.117-0.306) to extensive farms (OR= 0.558; 95% CI: 0.354-0.882). Other significant risk factors resulted to be possibility to graze, kind of feeding, water source and presence of other animal species (Table 3). In the final multi-variable model, only three variables and two interactions were entered (Table 4).

Table 3 - Potential risk factors for *T. gondii* seropositivity in goats by univariate analysis.

Variable	Category	n	Prevalence (%)	Odds ratio	95 % CI	p-value
Sampling area	Eastern (reference)	16	50.0%			
	Southern	61	29.2%	0.412	0.194-0.876	**0.021**
	Western	121	51.9%	1.080	0.516-2.262	0.838
Altitude	Continuous variable	474		1.000	1.000-1.001	0.103
Breed	Nera di Verzasca (reference)	56	44.4%			
	Alpine	37	38.9%	0.797	0.464-1.371	0.413
	Crossbreed	74	48.7%	1.186	0.738-1.905	0.481
	Saanen	31	30.7%	0.554	0.319-0.959	**0.035**
Gender	Male (reference)	7	53.8%			
	Female	191	41.4%	0.606	0.201-1.833	0.375
Age	Continuous variable	474		1.011	1.004-1.017	**0.001**
Species on farm	Onlygoats (reference)	160	39.4%			
	Goats+Sheep	38	55.9%	1.396	1.077-1.808	**0.012**
Number of animals on farm	Continuous variable	474		0.998	0.997-0.999	**0.0001**
Rearing system	Semi-intensive (reference)	93	60.0%			
	Extensive	67	45.6%	0.558	0.354-0.882	**0.012**
	Intensive	38	22.1%	0.189	0.117-0.306	**0.0001**
Grazing	No (reference)	74	31.0%			
	Yes	124	52.8%	2.491	1.712-3.625	**0.0001**
Feeding concentrate	No (reference)	97	36.9%			
	Yes	101	47.9	1.571	1.087-2.271	**0.016**
Water source	Municipal water (reference)	74	31.0%			
	River	124	52.8%	2.491	1.712-3.625	**0.0001**
Presence of other species	No (reference)	81	35.1%			
	Yes	117	48.1%	1.705	1.178-2.467	**0.005**
Presence of cats	No (reference)	16	50.0%			
	Yes	64	48.1	0.928	0.429-2.007	0.849
Purchase of spare breeding animals	No (reference)	52	52.5%			
	Yes	47	43.9	0.708	0.409-1.226	0.218

Statistically significant variables are indicated by bold typing

Table 4 - Potential risk factors associated with *T. gondii* seropositivity in goats using multivariate multi-level modeling.

Variable	Category	n	Prevalence (%)	Odds ratio	95 % CI	p-value
Breed	Nera di Verzasca (reference)	70	44.4			**0.0001**
	Alpine	58	38.9	0.797	0.464-1.371	0.413
	Crossbreed	78	48.7	1.204	0.509-2.845	0.673
	Saanen	70	29.6	0.251	0.094-0.669	**0.006**
Rearingsystem	Semi-intensive (reference)	62	60			**0.019**
	Extensive	80	45.5	0.285	0.119-0.684	**0.005**
	Intensive	134	22	0.669	0.251-1.781	0.421
Age	Continuous variable	474		1.020	1.012-1.028	**0.0001**
Rearing system * Number of animals on farm	Semi-intensive (reference)	62				**0.002**
	Extensive	80		0.999	0.990-1.009	0.898
	Intensive	134		1.003	0.992-1.015	0.565
Number of animals on farm * breed	Nera di Verzasca (reference)	70				**0.001**
	Alpine	58		.996	0.986-1.005	0.376
	Crossbreed	78		1.001	0.992-1.010	0.820
	Saanen	70		1.003	0.994-1.013	0.478

Statistically significant variables are indicated by bold typing

For sheep, univariate analysis showed several significant risk factors (Table 5). Sampling area was by far one of strongest predictors of *T. gondii* infection in a sheep flock. Odds for a sheep from the south-eastern area of being diagnosed seropositive to *T. gondii* were 3.256 times higher than for a sheep from the western area (OR= 3.256; 95% CI: 1.985-5.539). Seroprevalence increased in small measure with altitude of farms (OR=1.001; 95% CI: 1.001-1.002). Breed resulted to be a further significant risk factor, being the crossbreeds more infected than the other two breeds. As for goats, age was a risk factor, where seropositivity increased with the increasing of age (OR= 1.019; 95% CI: 1.011-1.026). The higher the number of animals in a farm, the higher their risk of being infected (OR= 1.001; 95% CI: 1.001-1.002). However, transhumant herds in comparison with semi-intensive ones appeared being at higher risk of *T. gondii* infection (66.8% *vs.* 38.4%). Unlike other species, the presence of goats in a farm had no effects on sheep seroprevalence, (OR= 2.197; 95% CI: 1.495-3.229) (Table 5). For sheep, variables such as grazing, feeding concentrate and water source were not considered, being monitored sheep bred under same conditions. For these animals, the final model included variables such as sampling area, altitude, age, and interaction between rearing system and number of animals on farm (Table 6).

Both for goats and sheep, variables associated to farm management, such as water source and purchase of spare breeding animals, did not enter the final model. Moreover, the presence of cats on farms or at grazing seemed to influence the seroprevalence in our survey.

Table 5 - Potential risk factors for *Toxoplasma gondii* seropositivity in sheep by univariate analysis.

Variable	Category	n	Prevalence (%)	Odds ratio	95 % CI	p-value
Sampling area	Western (reference)	29	35.4%			
	East-southern	269	64.0%	3.256	1.985-5.339	**0.000**
Altitude	Continuous variable			1.001	1.001-1.002	**0.000**
Breed	Bergamasca (reference)	105	52.2%			
	Crossbreed	171	71.8%	2.333	1.571-3.465	**0.000**
	Merinos	22	34.9%	0.491	0.273-0.833	**0.017**
Gender	Male (reference)	30	48.4%			
	Female	268	60.9%	1.662	0.975-2.834	0.062
Age	Continuous variable	502		1.019	1.011-1.026	**0.000**
Number of animals on farm	Continuous variable	502		1.001	1.001-1.002	**0.000**
Species on farm	Onlysheep (reference)	108	55.4%			
	Goats+Sheep	190	61.9%	1.308	0.909-1.883	0.148
Rearingsystem	Semi-intensive (reference)	48	38.4%			
	Extensive	19	61.3%	2.540	1.133-5.696	**0.024**
	Transhumant	231	66.8%	3.222	2.108-4.925	**0.000**
Presence of otherspecies	No (reference)	72	46.1			
	Yes	226	65.3	2.197	1.495-3.229	**0.000**
Presence of cats	No (reference)	190	64.2%			
	Yes	36	72.0%	1.435	0.740-2.780	0.285

Statistically significant variables are indicated by bold typing

Table 6 - Potential risk factors associated with *T. gondii* seropositivity in sheep using multivariate multi-level modeling.

Variable	Category	N	Prevalence (%)	Odds ratio	95 % CI	p-value
Sampling area	Western (reference)	29	35.4			**0.000**
	East-southern	269	64.0	7.782	2.139-2.316	**0.002**
Altitude	Continuousvariable	502		0.999	0.998-1.000	**0.003**
Age	Continuousvariable	502		1.011	1.002-1.021	**0.022**
Management × Number of animals on farm	Semi-intensive (reference)					**0.000**
	Extensive	115	66.7	1.014	1.007-1.022	**0.000**
	Transhumant	77	38.4	1.001	1.001-1.002	**0.000**

Statistically significant variables are indicated by bold typing

1.1.3. *Toxoplasma gondii* infection: Discussion

In the last years, increasing attention has been brought to *T. gondii* infection. In fact, the European Food Safety Authority (EFSA) has indicated toxoplasmosis as one of the most important parasitic zoonosis due to its high human incidence and published a recent scientific opinion clearly stating the need for investigation on its occurrence both in humans and animals in Europe (EFSA 2007). Epidemiological data on *T. gondii* infections in animals for human consumption are not regularly collected and current lack of standardization of diagnostic techniques and protocols should be taken into account when comparing seroprevalence data (Tenter et al. 2000). The present study aimed to update information on *T. gondii* infection in small ruminants in northern Italy and revealed that seroprevalence of anti-*Toxoplasma* antibodies was high both in goats and sheep at individual (goat=41.7%, sheep=59.3%) and at farm level (goat=96.6%, sheep=87.5%). Seroprevalence showed higher here than in animals tested in other Italian regions, but in a previous survey similar values had been reported in sheep from the same area (Gaffuri et al. 2006). High seroprevalence was also found in different European countries such as the Netherlands, Portugal, France, Switzerland, Romania, Greece, and Spain, and huge variations in the prevalence of *T. gondii* ranging from 18.5% to 52.8% in goats and from 27.8% to 89% in sheep observed (Halos et al. 2010, Opsteegh et al. 2010, Berger-Schoch et al. 2011, Iovu et al. 2012, Tzanidakis et al. 2012, Garcia-Bocanegra et al. 2013, Lopes et al. 2013). Higher seroprevalence recorded in sheep rather than in goats is consistent with values reported in previous studies that considered both species reared in the same areas (Masala et al. 2003, Tzanidakis et al. 2012, Garcia-Bocanegra et al. 2013, Lopes et al. 2013).

Further, in the present study a large number of sampled animals had a titre of only 1:64 or 1:128 both in goats and in sheep suggesting that most animals were in the chronic phase of infection.

In both species, seroprevalence was positively correlated to age, as already stated in previous studies (Spisak et al. 2010). However, a difference in the proportion of seropositive small ruminants was found when the age of animals was separately compared for both species. In sheep, seroprevalence regularly goes up with the increasing of age, with the highest seroprevalence in animals > 6 years old. Conversely, in goats the percentage of seropositive animals irregularly varies with age, showing that their antibody response could be probably weaker than in sheep aged from 5years onward. Such difference in seroprevalence may be explained by a difference in their immune response. In fact, several studies previously illustrated that both acquisition and expression of immune responses against gastrointestinal nematodes are less efficient in goats than in sheep, though few studies were published on differences in susceptibility to toxoplasmosis of the two species (Innes 1997). Besides, other external factors such as farm management or feeding behavior could account for this discrepancy (Hoste et al. 2010).

Analysis of risk factors showed that Saanen goats presented the lowest risk of being infected and crossbreeds the highest. Differently, Lopes et al. (2013) reported a lower seroprevalence value in crossbreeds compared with defined-breed goats. In northern Italy, noticeably Saanen goats are bred mostly in intensive farms presenting the lowest prevalence (30.7%), so differences reported in this survey may be associated to differences in rearing systems and not in breeds.

In sheep, altitude is a significant risk factor, being positively associated to seroprevalence that increases in hilly areas between 300 and 1000 ma.s.l where transhumance toward lowlands in the winter is still practiced. Altitude is frequently reported as a risk factor associated to toxoplasmosis in different countries and is related to environmental conditions and different grazing strategies (Skjerve et al. 1998, Gebremedhin et al. 2013). Moreover, in the south-eastern area where most transhumant sheep herds are, the highest seroprevalence were recorded in these animals.

Rearing system was indeed a very important risk factor associated to the infection, both in goats and sheep: extensive or semi-intensive farms and transhumant herds were the breeding management at higher risk of toxoplasmosis. Intensive farms in northern Italy may have a high level of hygiene preventing *T. gondii* oocysts from spreading throughout their facilities. On the contrary, semi-intensive goat farms, often represented by small family businesses, may have an inadequate hygienic standard and consequent spread of *T. gondii* oocysts within their animals. Extensive farms or transhumant herds may be more exposed to cats in the environment or to contaminated stagnant pools, even though oocysts may be more dispersed in the environment. Nevertheless, a previous survey carried out in Greece recorded the highest infection prevalence in intensive farms

(Tzanidakis et al. 2012) highlighting differences in the management of the farms. In intensive farms, animals could possibly be more exposed to contaminated feed and farm facilities under intensive or semi-intensive conditions may provide shelter to various hosts of *T. gondii* (such as cats and rodents) which might be involved in the spread of infection. Interestingly, in this survey the presence of cats on farms or on the grazing sites did not represent a meaningful risk factor associated to the infection, differently from other surveys (Cenci-Goga et al. 2013). Farmers might not notice stray cats on their farms or on the grazing sites, which can account for the contamination of pastures or feed or water sources, according to Tzanidakis et al. (2012).

Another significant variable was farm size, connected to rearing system. Regarding goats, the number of seropositive animals was negatively associated with the size of the flock in semi-intensive or extensive farms; therefore, seroprevalence was higher in smaller flocks. Again, small family businesses showed higher prevalence than large intensive farms. In sheep, conversely, seroprevalence was positively correlated to the herd size; large transhumant herds possibly contribute to the maintenance of the infection within the animals. Vesco et al. (2007) reported a similar situation in sheep reared in Sicily region; on the contrary, data reported by Cenci-Goga et al. (2013) showed that in sheep reared in Tuscany seroprevalence is negatively correlated to size farm, corresponding to a major infection in smaller farms as registered in our goats. Therefore, different seroprevalence values in both species may correspond to different production systems.

1.1.4. *Neospora caninum* infection: Results

According to ELISA, at individual level 57.4% (238/414; 52.6-62.1%, 95% CI) of goats and 59.5% (255/428; 54.8-64.1%, 95% CI) of sheep resulted positive. At farm level, antibodies to *N. caninum* were found in 25 (89.2%; 77.7-100%, 95% CI) out of 28 goat flocks, whereas all 19 (83.1-100%, 95% CI) sampled sheep farms presented at least one seropositive animal. Considering data obtained with Western Blot analysis both sheep and goats positive sera recognized all immnunodominant antigens described in each antigenic area (Fig. 3). Only 24 goats (5.7%; 3.5-8%, 95% CI) and 83 sheep (19.3%; 15.5-23%, 95% CI) were confirmed positive. Consequently, the seroprevalence at flock level decreases to 32.1% (9/28; 14.8-49.3%, 95% CI) and 89.4% (17/19; 75.56-100%, 95% CI) for goats and sheep, respectively (Table 7).

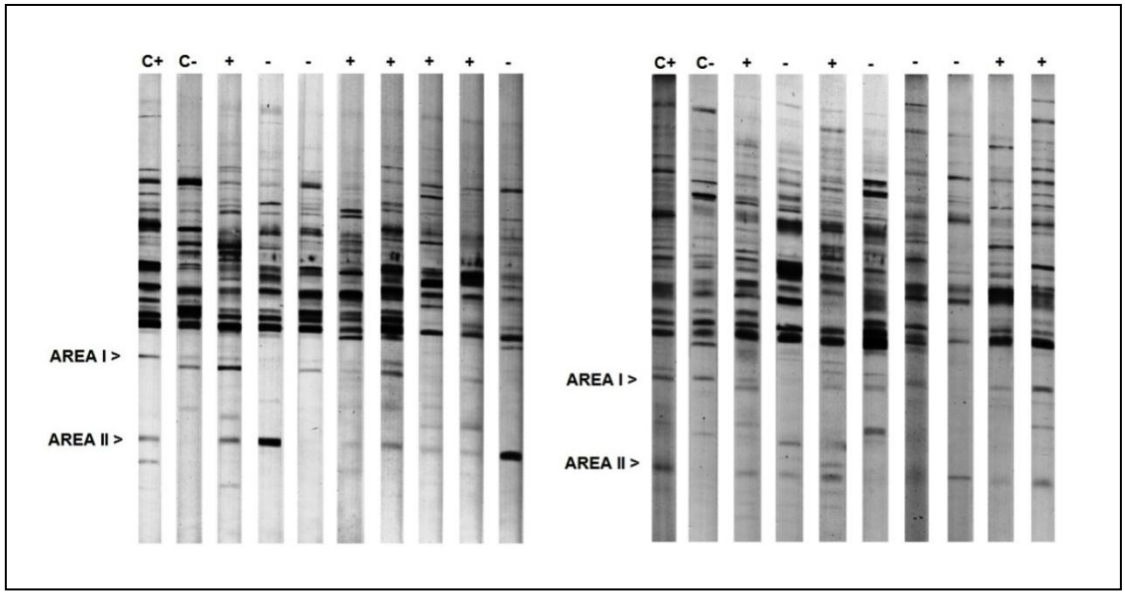

Fig. 3 - Pattern of recognition of *N. caninum* tachyzoite antigens by sera from naturally infected goats (on the left) and sheep (on the right) by Western Blot.

Table 7 – *N. caninum* seroprevalence values by ELISA and WB for goats and sheep at flock and individual level.

	Flock level			Individual level		
	No. examined	ELISA P% (95% CI)	WB P% (95% CI)	No. examined	ELISA P% (95% CI)	WB P% (95% CI)
Goats	28	89.2 (77.7-100)	32.1 (14.8-49.3)	414	57.4 (52.6-62.1)	5.7(3.5-8)
Sheep	19	100 (83.1-100)	89.4 (75.5-100)	502	59.3 (54.8-64.1)	19.3 (15.5-23)

Spatial distribution of positive flocks is represented in Fig. 4.

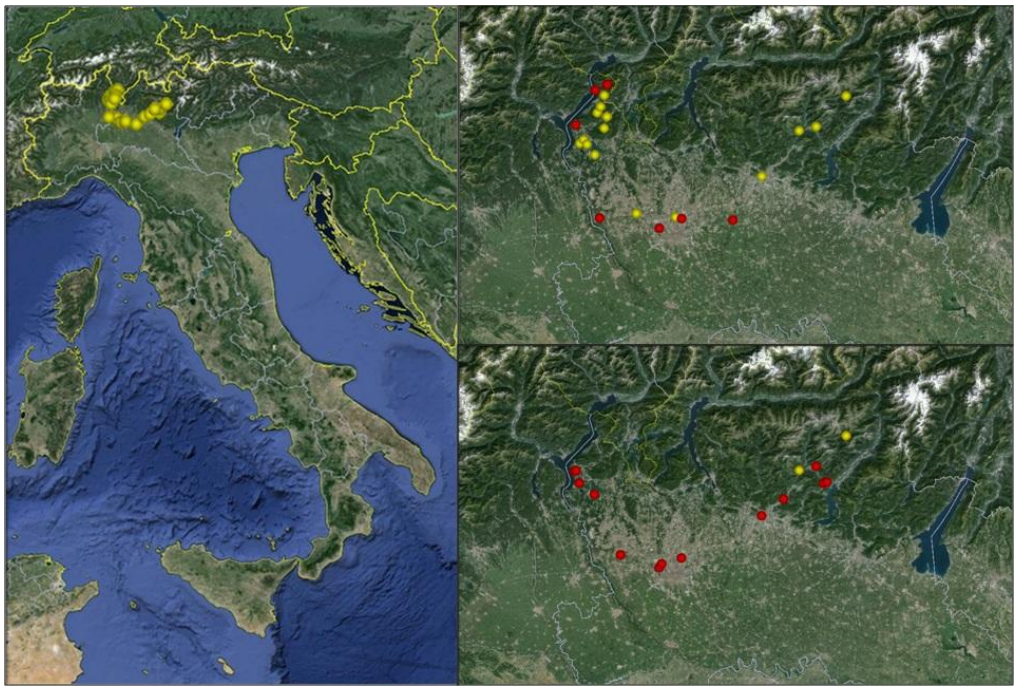

Fig. 4 - Spatial distribution of sampled farms; on the right, the upper goats, the lower sheep. Red farms resulted positive to *N. caninum*.

The true positive samples presented ELISA OD values ranging from the cut-off to 2.894 (Fig. 5). Particularly, goats showed lower values than sheep, with most animals (16 out of 24 positive) with OD values comprised between the cut-off and 0.6. On the contrary, most of ovine samples (40 out of 83 positive) presented OD values comprised between 0.6 and 0.9.

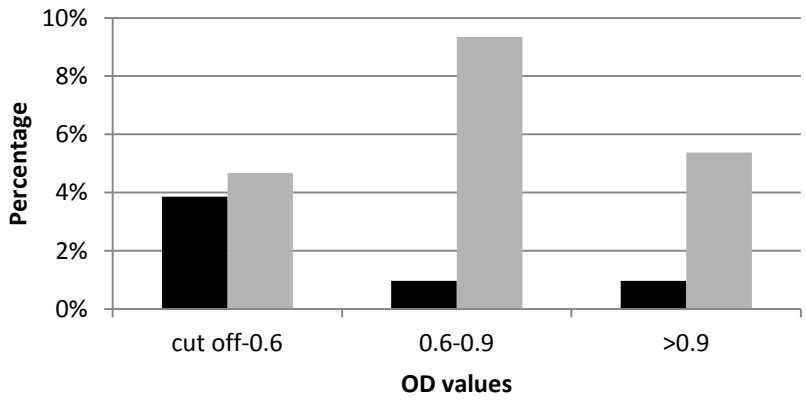

Fig. 5 - ELISA OD values in samples confirmed positive to *N. caninum* by WB (goats in black, sheep in grey).

In positive flocks, the number of seropositive animals varied according to the host species and management system. Regarding goats, only farm “8” and “9” of the nine positive farms are

intensive ones with only one (2.6%) and two (1.9%) positive animals, respectively (Fig. 3). In extensive and semi-intensive flocks more animals were found positive, with an intra-flock prevalence ranging from 2.7% to 25% (mean 13.4%). Considering sheep, only two farms did not show any positive animals corresponding to two transhumant flocks. The highest intra-flock seroprevalence values were registered in semi-intensive or extensive farms (min-max: 7.7-62.5%; mean 32.4%), whereas transhumant flocks showed lower values (min-max: 6.7-25%, mean 13.4%) (Fig. 6-7).

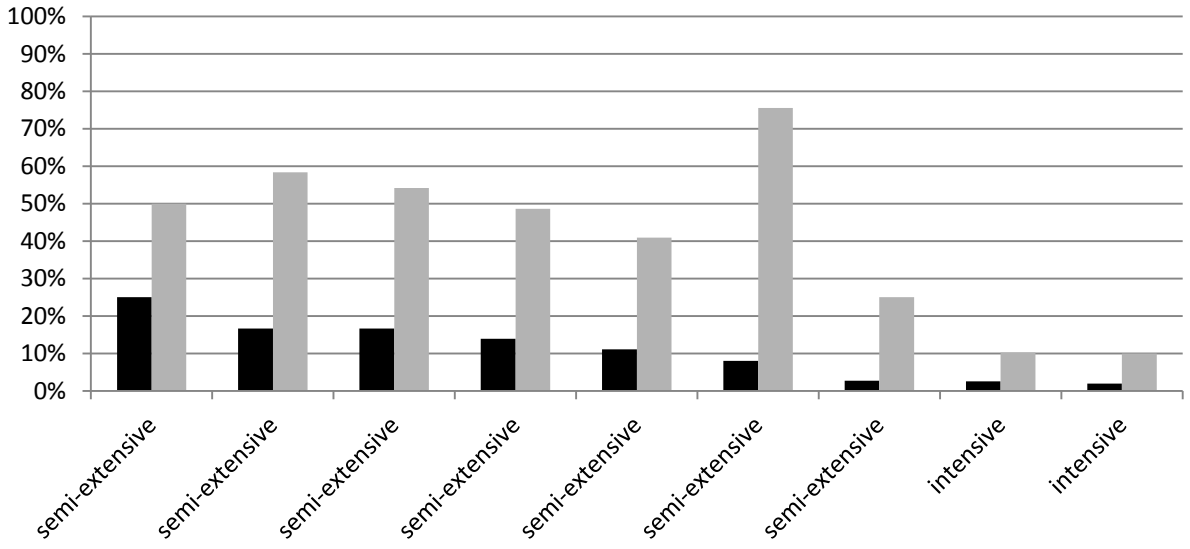

Fig. 6 - Positive correlation between *N. caninum* (black) and *T. gondii* (grey) intra-herd seroprevalence in *N. caninum* positive caprine farms.

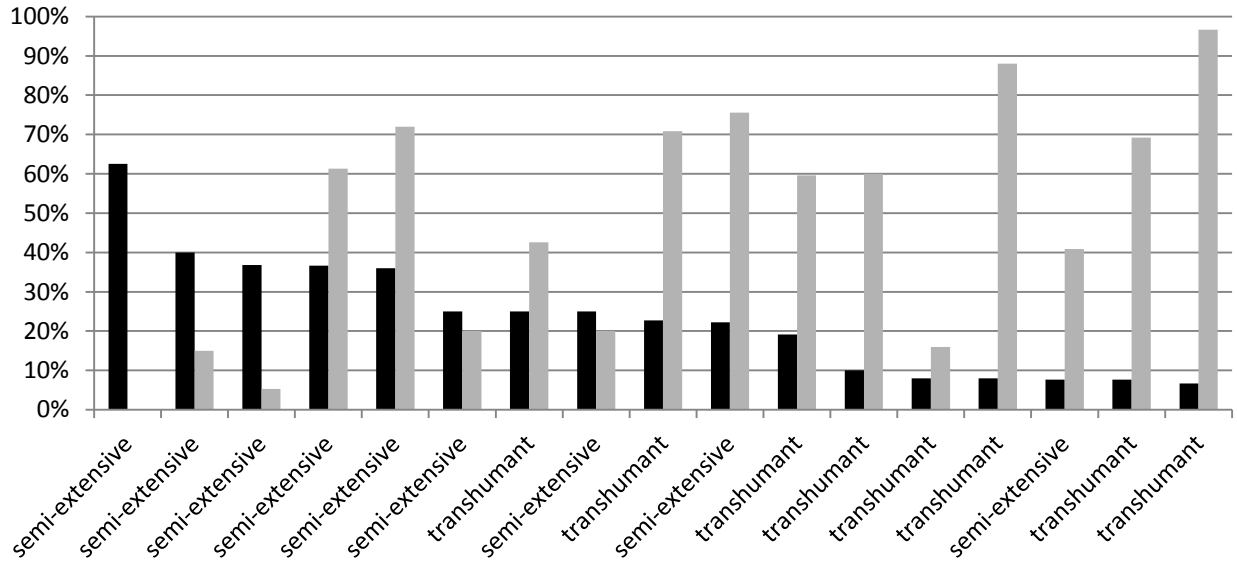

Fig. 7 - Positive correlation between *N. caninum* (black) and *T. gondii* (grey) intra-herd seroprevalence in *N. caninum* positive ovine farms.

The analysis of potential risk factors was separately conducted for the two species since seroprevalence was significantly higher in sheep than in goats (Pearson's chi-square, p=0.0001). Results obtained from risk factor univariate analysis for goats are given in Table 8. Sampling area did not result a significant variable; however, it is interesting to notice the absence of positive goat flocks in the province of Bergamo (Fig. 4). Breed represents a significant risk factor, being Alpine (P=2.22%; OR=0.161) and crossbreed (P=2.82%; OR=0.205) less seropositive to *N. caninum* than Saanen (P=4.92%; OR=0.366) and Nera di Verzasca (P=12.4%). Age does not represent a risk factor for the infection, varying age of positive animals from 8 months to 7 years (mean 40 months) (Fig. 8).

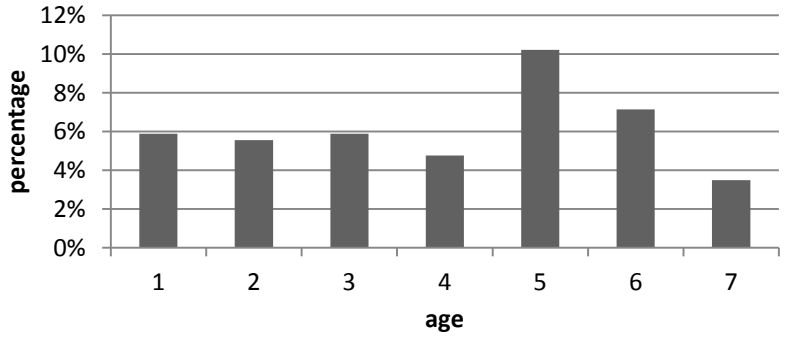

Fig. 8 - Percentage of goats seropositive to *N. caninum* according to different class of age (expressed in years).

Rearing system is associated to the infection with semi-extensive farms more at risk (OR=3.515) than intensive ones; also the farm size is a significant variable, being small flocks more at risk than bigger ones (p=0.023; OR=0.996). Altitude of farm resulted to be another risk factor associated to the infection, being positively related to the infection (p=0.047; OR=1.001). Goats having access to graze (P=8.11%; OR=2.735) and to river (P=8.11%; OR=2.735) for water are more at risk than those kept indoor. The purchase of spare breeding animals did not result a significant variable as well as the presence of dogs or other species in farm or at grazing. Goats presenting antibodies against *Toxoplasma gondii* resulted more at risk of infection by *N. caninum* (P=9.25%; OR=2.968) than the negative ones (P=3.32%). In the final multivariate model, only the variable "breed" remained in the model (Table 9).

Table 8 - Risk factors associated with *N. caninum* infection in goats according to univariate analysis.

Variable	Category	No. examined	No. positives	Prevalence (%)	Odds ratio	95 % CI	p-value
Sampling area	Varese (reference)	164	17	10.37			0.073
	Bergamo	32	0	0.00	0.000	0-0	0.998
	Milano	201	7	3.48	0.348	0.141-0.86	0.022
Altitude	Continuous variable				1.001	1-1.003	**0.047**
Breed	Nera di Verzasca (reference)	121	15	12.40			**0.008**
	Alpine	90	2	2.22	0.161	0.036-0.721	**0.017**
	Crossbreed	142	4	2.82	0.205	0.066-0.635	**0.006**
	Saanen	61	3	4.92	0.366	0.102-1.315	0.123
Age	Continuous variable				0.996	0.981-1.011	0.586
Gender	Female (reference)	403	24	5.96			
	Male	11	0	0.00	0.000	0.000-0.000	0.999
Number of animals on farm	Continuous variable				0.996	0.993-0.999	**0.023**
Rearing system	Intensive (reference)	165	4	2.42			
	Extensive + semi-intensive	249	20	8.03	3.515	1.179-10.479	**0.024**
Purchase of spare breeding animals	No (reference)	59	2	3.39			
	Yes	104	11	10.58	3.371	0.721-15-76	0.123
Grazing	No (reference)	192	6	3.13			
	Yes	222	18	8.11	2.735	1.063-7.038	**0.037**
Water source	Municipal water (reference)	192	6	3.13			
	River	222	18	8.11	2.735	1.063-7.038	**0.037**
Presence of dogs	No (reference)	25	2	8.00			
	Yes	174	16	9.20	1.165	0.251-5.398	0.846
Presence of otherspecies	No (reference)	347	21	6.05			
	Yes	67	3	4.48	0.853	0.459-1.585	0.615
***T. gondii*(IFAT)**	No (reference)	241	8	3.32			
	Yes	173	16	9.25	2.968	1.24-7.102	**0.015**

Statistically significant variables are indicated by bold typing

Table 9 - Risk factors associated with *N. caninum* infection in goats according to multivariate analysis.

Variable	Category	No. examined	No. positives	Prevalence (%)	Odds ratio	95 % CI	p-value
Breed	Nera di Verzasca (reference)	121	15	12.40			**0.008**
	Alpine	90	2	2.22	0.161	0.036-0.721	**0.017**
	Crossbreed	142	4	2.82	0.205	0.066-0.635	**0.006**
	Saanen	61	3	4.92	0.366	0.102-1.315	0.123

Statistically significant variables are indicated by bold typing

Considering the univariate analysis on sheep data (Table 10), differences among sampling areas resulted significant, being sheep from Bergamo (P=12.71%; OR=0.216) less at risk than those from Milan (P=28%; OR=0.576) and Varese (P=40.32%). Seroprevalence decreases with the increasing of altitude (Table 5). Breed is a risk factors associated to *N. caninum*, resulted Merinos sheep more infected (P=40.32%; OR=3.443) than Bergamasca (P=15.2%; OR=0.913) and crossbreed (P=16.41%). Gender and age were not associated to the infection; the age of seropositive sheep ranged from 7 months to 7 years (mean 4 years) (Fig. 9).

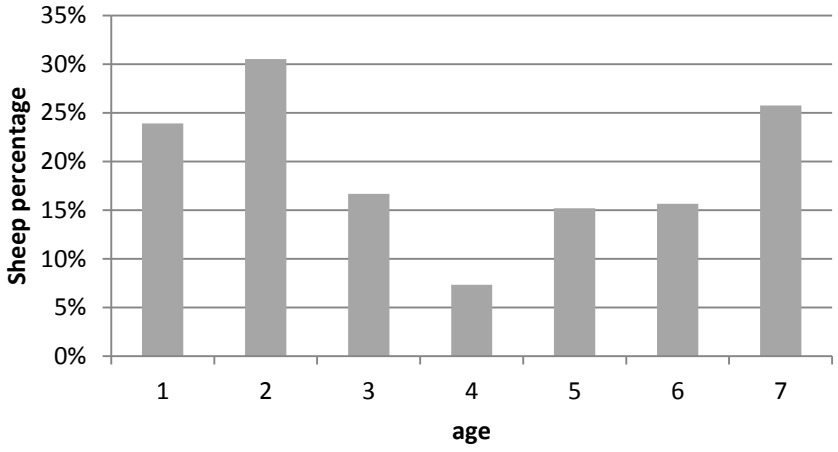

Fig. 9 - Percentage of sheep seropositive to *N. caninum* according to different class of age (expressed in years).

The presence of goats in farm influenced the seroprevalence being mixed flocks less at risk (P=13.62%; OR=0.319) than those with only sheep (P=33.07%). Transhumant flocks appeared to be less exposed to *N. caninum* (P=12.54%) than extensive (P=36.67%; OR=4.037) or semi-intensive ones (P=32.43%; OR=3.347). Similarly, the flock size result a significant risk factor, increasing seroprevalence with the decrease of the number of animals. The possibility to graze as well as the water source and the presence of dogs did not appear to influence the positivity to *N. caninum*. Finally, sheep positive to *T. gondii* resulted to be less at risk of infection by *N. caninum*

(P=15.41%; OR=0.523) than the negative ones (P=25.42%). The multivariate model contained only the "rearing system" variable (Table 11).

Many of the variables included in the models did not result statistically significant. Of particular interest, the variable "age" did not influence the seroprevalence neither in sheep or goats, although seroprevalence seems distributed in age groups differently in sheep and goats (Fig. 8Fig. 9). Neither the presence of dogs in farm or at grazing resulted to be significant risk factors both in ovine and caprine models. For goats, other variables did not resulted statistically significant: sampling area, gender, the purchase of spare breeding animals, and the presence of other species in farm. For sheep, gender, possibility to graze and water resource did not influence the seropositivity to *N. caninum*.

Table 10 - Risk factors associated with *N. caninum* infection in sheep according to univariate analysis.

Variable	Category	No. examined	No. positives	Prevalence (%)	Odds ratio	95 % CI	p-value
Sampling area	Varese (reference)	62	25	40.32			**0.000**
	Bergamo	291	37	12.71	0.216	0.117-0.398	**0.000**
	Milano	75	21	28.00	0.576	0.281-1.177	0.130
Altitude	Continuousvariable				0.999	0.998-1	**0.002**
Breed	Meticcio (reference)	195	32	16.41			**0.000**
	Bergamas	171	26	15.20	0.913	0.520-1.605	0.753
	Merinos	62	25	40.32	3.442	1.827-6.484	**0.000**
Age	Continuousvariable				0.996	0.987-1.006	0.459
Gender	Female (reference)	369	74	20.05			
	Male	59	9	15.25	,718	0,337-1,525	0.388
Presence of other species	No (reference)	127	42	33.07			
	Yes	301	41	13.62	0.319	0.195-0.524	**0.000**
Number of animals on farm	Continuousvariable				0.999	0.999-0.999	**0.000**
Rearingsystem	Transumant (reference)	287	36	12.54			
	Extensive + semi-intensive	141	47	33.33	3.486	2.126-5.717	**0.000**
Grazing	No (reference)	18	4	22.22			
	Yes	410	79	19.27	0.835	0.268-2.607	0.757
Water source	Municipal water (reference)	18	4	22.22			
	River	410	79	19.27	0.835	0.268-2.607	0.757
Presence of dogs	No (reference)	18	4	22.22			
	Yes	321	48	14.95	0.615	0.194-1.949	0.409

Statistically significant variables are indicated by bold typing

Table 11 - Risk factors associated with *N. caninum* infection in sheep according to multivariate analysis.

Variable	Category	No. examined	No. positives	Prevalence (%)	Odds ratio	95 % CI	p-value
Rearing system	Transumant (reference)	287	36	12.54			
	Extensive + semi-intensive	141	47	33.33	3.486	2.126-5.717	**0.000**

Statistically significant variables are indicated by bold typing

1.1.4. *Neospora caninum* infection: Discussion

The current study provided serological data on *N. caninum* infection among sheep and goats in northern Italy. In the study, seroprevalence was higher in sheep than goats at individual, flock and intra-flock levels. At individual level, a number of seroreactors significantly higher was found in sheep than in goats (p<0.0001) according to previous surveys (Bartova and Sedlak 2012, Nasir et al. 2012a, Diakou et al. 2013, Topazio et al. 2014). At flock level, seroprevalence was higher in sheep than in goats (89.4% *vs.* 32.1%, respectively) as previous observed by several authors when neosporosis was investigated simultaneously in both species (Abo-Shehada and Abu-Halaweh 2010, Nasir et al. 2012a, Diakou et al. 2013). Diakou et al. (2013) according to Dubey and Lindsay (1996) speculated that the higher prevalence of infection among sheep may be correlated to a greater susceptibility of this species;. The same authors suggested that the difference in seroprevalence observed in sheep and goats could be due to different feeding behavior. Sheep, generally considered grazers, are more exposed to the risk of getting infected by pathogens found close to the ground, such as the infective stage of Apicomplexa protozoa (oocysts). On the contrary, goats are considered browsers, and as a consequence to their feeding habit exhibit lower levels of anti-*Neospora* antibodies than sheep. However, goats are able to graze similarly to sheep; it was indeed demonstrated that goats have the same risks as sheep to acquire gastrointestinal nematodes infection through the ingestion of grass contaminated by parasites' eggs (Hoste et al. 2010). Goats could therefore have the same probability of ingest *N. caninum* oocysts acquiring the infection. Moreover, the hypothesis that an immune-inadequacy exists regarding the protozoan infection in goats is worth investigating.

Differences between sheep and goats could be further observed comparing the distribution pattern of seroprevalence values in relation with age, presenting young sheep and adult goats the highest prevalence values, respectively (Fig. 8Fig. 9). It could indicate a possibility for neosporosis to be transmitted vertically in sheep more frequently than in goats, although further studies are necessary.

However, the different observed pattern could be also related to differences in kids and lambs management. In northern Lombardy, kidding usually occurs on farm in winter and young goats only graze the next season. On the contrary, lambing occurs more frequently at pasture or in the paddock where sheep are only recovered at night. Therefore, lambs may become infected through the ingestion of *N. caninum* oocysts earlier than goats. The transplacental transmission is retained the major mode of transmission of *N. caninum* for cattle (Barr et al. 1993, Schares et al. 1998, Hietala and Thurmond 1999) whereas little is known for small ruminants. A few studies, concerning the detection on *N. caninum* in aborted fetuses, indicate that transplacental transmission seems occur both in sheep and goats even if frequency and etiology of abortion should be further solved (Eleni et al. 2004, West et al. 2006, Masala et al. 2007, Abo-Shehada and Abu-Halaweh 2010).

Goats and sheep showed to have different environmental and managerial variables linked to neosporosis. At the intra-flock level, the major number of seroreactors to *N. caninum* was found in sheep flocks reared under semi-extensive systems. Similarly, in goats the highest intra-flock prevalence (24%) was measured in a flock with semi-extensive system. For goats, the farm size measured in number of animals seems to be linked inversely to the infection, being smaller farm more at risk in comparison to farms with a huge quantity of goats. Large caprine intensive farms, located traditionally in northern Italy flatland, even if not free to *N. caninum* infection, presented indeed quite low seroprevalence values. Abortions and reproductive diseases could be limited to some clinical outcome and farms may face these disorders culling not-productive animals and replacing them with not infected ones. Traditional kind of farming (i.e. semi-extensive farms) seems to be more at risk of infection in comparison to intensive one, usually at a higher standard level of technology and hygiene: an example is the access to the placenta for dogs or to other canids, facilitating the spread of *N. caninum* infection. Concerning semi-extensive system, sheep and goats in winter received food supplementation of grain and forages produced in or out the farm that was kept in storehouses easily accessible to dogs posing this practice an important risk of infection (Dubey and Schares 2011). Otherwise, sheep from transhumant flocks appeared to be less exposed to *N. caninum* infection than sheep from flocks reared under other breeding systems. The transhumance is still practiced in northern Italy and sheep are traditionally moved from alpine pastures to lowlands in winter; however in our study most of the transhumant flocks surveyed were moved within different areas located in lowlands characterized by intensive agriculture and dairy farming where sheep are used to graze the grass in meadows. Then, the lowest prevalence found in these flocks could be related to different habitat features with a very low number of wild species and dogs. Further, the transhumant flocks respect to the others consisted of a large number of sheep (>1000 head for flock) and other domestic species, as goats, dogs and cattle, were scarce or absent.

Considering other risk factors, the possibility to graze and the access to natural stagnant water source represent risks for *N. caninum* infection in goats. Further, in the autochthonous breed "Nera di Verzasca" the highest seroprevalence in comparison to the other breeds included in the survey (Alpine, Saanen and crossbreed) was registered. In sheep, Merinos sheep seem to be more at risk than Bergamasca and crossbreed and the contemporary presence of goats within ovine flocks represents a risk factor for neosporosis: it could again be explained by the traditional kind of management, since mixed flocks in northern Italy are more often reared under semi-extensive conditions.

Thus, the risk of *N. caninum* infection appears to be more severe for small ruminants having regularly access outdoor. At grazing, both sheep and goats could be more exposed to the risk of close contact to the definitive host shedding oocysts. However, in our study the presence of dogs in farm or at grazing did not represent a risk factor, contrasting with the conclusions of Abo-Shehada and Abu-Halaweh (2010) but in agreement with findings of Machado et al. (2011) and Castaneda-Hernandez et al. (2014). Dogs are among the proven definitive hosts shedding oocysts of the protozoan (Dubey and Schares 2011). Actually, a few species of wild canids are considered able to shed oocysts; and more recently high seroprevalence values were detected in free-ranging dogs from Aboriginal communities suggesting that postnatal infection is common in these animals (King et al. 2012). On the contrary, red foxes are proved to be infected by *N. caninum* but oocysts were not detected in any feces (Schares et al. 2002, Constantin et al. 2011). It is known that naturally infected dogs shed a relatively small number and frequency of shedding decreases with the age and the immune status of the host (Schares et al. 2005). Therefore, some authors suggest that in small ruminants postnatal transmission, involving oocysts, occurs rarely in nature; it suggests, again, vertical transmission of the parasite the major way of infection for ruminants (Hall et al. 2005). However, the role of environmental contamination by oocysts in the infection by *N. caninum* should be further investigated considering that the protozoan infection resulted especially spread among small flocks, with autochthonous caprine breed grazing in areas with high elevation where many species of wildlife are largely present. Up to now, serosurveys stated the antibodies anti-*N. caninum* were detected in a wide range of domestic and wild animals: cats, pigs, rodents, lagomorphs, mustelids, avians, red foxed and wild ruminants (Dubey and Schares 2011). However, neosporosis sylvatic cycles and the role of wildlife as reservoir of *N. caninum* for domestic ruminants is still to be clarified (Almeria et al. 2007, Billinis 2013).

Differences between sheep and goats on *N. caninum* infection regard also its relationship with *T. gondii*. In sheep flocks, *N. caninum* prevalence decreased with the increasing of *T. gondii* seroprevalence, whereas in goat flocks *T. gondii* seroprevalences were higher and seem to vary as

the *N. caninum* prevalence changes (Fig. 6Fig. 7). Both in sheep and goats the relationship between *N. caninum* and *T. gondii* could be also due to management factors. In goats the highest seroprevalence values for both protozoa were found in small flocks, maybe suggesting that poor hygienic measures may facilitate the maintaining of infections and the horizontal transmission, in addition to vertical one, could play a role in the spread and maintaining of the infections. On the contrary, in large sheep transhumant flocks the lowest percentage of animals with antibody anti-*N. caninum* and the highest percentage of animal with antibody anti-*T. gondii* was found. Apparently, different risk factors seem implied in the spread and maintaining of *N. caninum* and *T. gondii* infections in sheep breed in the study area. Large transhumant flocks appeared to be more at risk for toxoplasmosis, suggesting the maintenance of the infection through horizontal transmission. Vice-versa, in small familiar farms higher prevalence of *N. caninum* than *T. gondii* were registered: in this kind of rearing system, hygiene may not reach adequate standard. It is a common custom, indeed, to allow domestic dogs to enter the sheep-pen and to stay with the animals while grazing, maybe contributing the persistence of the infection in the farm. As reported above, neosporosis in sheep spreads well also through vertical way, whereas a horizontal transmission could be a major way of *T. gondii* infection. However, further studies are necessary to deepen interaction between the two pathogens (Figliuolo et al. 2004a, Figliuolo et al. 2004b).

2. Spatial analysis of *T. gondii* and *N. caninum* infections in small ruminants bred in Northern Italy

2.1. Introduction

Spatial analysis provides important additional information useful to study and interpret the geographical distribution and spread of diseases, since they allow increasing the comprehension of the association between disease processes and explanatory spatial variables. Moreover, geostatistical techniques can be used for spatial prediction (interpolation) between individual sample points (Graham et al. 2004). Further, climatic variables are important factors contributing to the spatial distribution of infectious diseases, since they may influence physiology and behavior of hosts and vectors (Thomson and Connor 2000).

Particularly in parasitology, spatial techniques found applications on the study of on zoonotic parasitic diseases (i.e. cystic echinococcosis, onchocerciasis, fasciolosis, schistosomiasis) or for the studies of vector-borne diseases: malaria, trypanosomiasis, tick-borne encephalitis (Thomson and Connor 2000, Zhou et al. 2009, Manfredi et al. 2011, Meurs et al. 2013, Cassini et al. 2014).

Among spatial analysis techniques, spatial scan statistic is particularly useful for geographical disease surveillance: it is based on the calculation of clusters when seroprevalence in an area is more frequent than expected. Clusters calculation contribute to study spatial distribution of an infection or disease and to evaluate their associated risk factors (Robinson 2000).

Considering *Toxoplasma gondii* and *Neospora caninum* infection in animals, few epidemiological studies were carried out utilizing geospatial tools (Fusco et al. 2007, Frössling et al. 2008, Klevar et al. 2010, Nogareda et al. 2013, Djokic et al. 2014). Therefore, epidemiological data on *T. gondii* and *N. caninum* infections in sheep and goats in Northern Italy were submitted to geospatial analysis and climatic data were considered and analyzed. The aim was to investigate on possible geographical or environmental factors influencing the distribution of the infections.

2.2. Materials and methods

Farms locations were plotted using Google Earth; from KML file, latitude and longitude were calculated using the free online tool software Kml2x (http://www.zonums.com/online/kml2x/).

Locations were then associated to infection (seropositivity) and subsequently imported to SaTScan™ 9.3 (Software for the spatial and space-time scan statistics, developed by M. Kulldorf, Harvard Medical School, Boston and Information Management Services Inc., Silver Spring, Maryland, USA. Available at http://www.satscan.org). To verify if cases of seropositivity were

randomly distributed in the study area or, on the contrary, were aggregated in geospatial clusters, Kulldorff's scan statistic in SaTScan™ was performed. Potential clusters are represented by moving circular windows with varying size (until a fixed maximum) used in the test. A maximum window size was fixed at 50% of the surveyed population (SaTScan™ User Guide for version 9.3).

To test whether the cases of infection were randomly distributed or there was a major risk of infection within the window, a likelihood ratio test was performed for each window basing on observed and expected number of cases inside and outside the window. The "most likely cluster" was the window with the highest likelihood; the *p*-value of the maximum likelihood ratio test statistic was obtained after 999 Monte Carlo replications and only statistically significant clusters ($p<0.05$) were considered (Kulldorff 1997).

Subsequently, to investigate whether risk factors linked to farm management could cause the creation of clusters or, anyway, interfere with statistically significant cluster in terms of size and location, a Bernoulli model was performed using multiple dataset (Kulldorff et al. 2007). According to both results obtained in the present and previous survey, to create multiple dataset we selected three risk factors for *T. gondii* and *N. caninum* in small ruminants: individual age, rearing system and farm size.

Individual age for spatial analysis was expressed in three categories: AGE1=<1year, AGE2=2-5years, AGE3=>5years. Further, two managerial variables were categorized as follow and analyzed together: "rearing system" (M1=extensive, M2= semi-extensive; M3=intensive for goats) and "farm size" (goats: S1=<100animals, S2= >100 animals; sheep: S1=<500 animals, S2= >500 animals). For sheep, transhumant herds were not included in the analysis, since a unique coordinate did not exist being animals moved during winter and springs; thus we cannot establish if the infection would be acquired in the winter period in the lowland or in summer in mountain pastures (Table 12).

Table 12 - Characteristics of the datasets used for the age-, management- and farm size-adjusted spatial analyses, for sheep and goats.

	***T. gondii* infection**				***N. caninum* infection**			
	Sheep		**Goat**		**Sheep**		**Goat**	
	n (overall)	**prevalence %**	**n (overall)**	**prevalence %**	**n (overall)**	**prevalence %**	**n (overall)**	**prevalence %**
AGE 1	9 (36)	25,0%	28 (99)	28,3%	13 (36)	36,1%	5 (99)	5,1%
AGE 2	33 (76)	43,4%	106 (229)	46,3%	21 (76)	27,6%	16 (229)	7,0%
AGE 3	21 (29)	72,4%	39 (86)	45,3%	13 (29)	44,8%	3 (86)	3,5%
M 1	18 (30)	60,0%	66 (143)	46,2%	11 (30)	36,7%	12 (143)	8,4%
M 2	45 (111)	40,5%	70 (106)	66,0%	36 (111)	32,4%	8 (106)	7,5%
M 3	-	-	37 (165)	22,4%	-	-	4 (165)	2,4%
S 1	61 (137)	44,5%	127 (215)	59,1%	46 (137)	33,6%	18 (215)	8,4%
S 2	2 (4)	50,0%	46 (199)	23,1%	1 (4)	25,0%	6 (199)	3,0%

AGE1=<1years; AGE2=2-4years; AGE3=>4years; M1=extensive; M2=semi-extensive; M3= intensive for goats; S1=<100 goats, <500sheep; S2=>100goats, >500sheep.

Subsequently, multivariate binary logistic regression analysis for *T. gondii* and *N. caninum* infections on climatic data were performed using as dependent variables the "presence/absence" (dichotomous variable) of antibodies anti-*T. gondii* and anti-*N. caninum*, respectively. A GIS database containing data on the study population, including farm location, serological results and climatic data, was generated using Quantum GIS version 2.4.0 (QGIS Development Team, 2014. QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org). Each animal was georeferenced individually.

As predictors, all variables obtained from the answers to the questionnaire were used; moreover, climatic data on were added. Climatic variables were obtained from WorldClim version 1.41 (http://www.worldclim.org) with a resolution of 30 arc-second (~ 1 km) (Hijmans et al. 2005). Climate data consisted of 4 variables which were expressed as mean monthly values averaged over a 50-year period (1950–2000): minimum, mean and maximum temperature divided per season (continuous variable, expressed in °C×10), rainfall divided per season (continuous variable, expressed in mm). In this analysis, transhumant sheep were considered: location and period of summer and winter pastures were known; therefore, data were inserted divided per months.

Mean temperature and maximum temperature were eliminated from the model since parameters in collinearity statistics: Tolerance ($1-R^2$) <0.5 and VIF (1/Tolerance) >2 indicated that collinearity between variables and "minimum temperature" was not acceptable. Interactions between "minimum temperature" and "rainfall" divided per season were also considered. Statistical analysis was performed with SPSS software (version 19.0; SPSS, Chicago, IL).

2.3. Spatial analysis of *T. gondii* infection: Results

Unadjusted spatial analysis highlighted a heterogenic distribution of cases of infection, creating clusters of infection ($p<0.05$) both for sheep and goats.

In the unadjusted spatial analysis on caprine data, five clusters were created: cluster 1 at low risk (coordinates: 45.506911 N, 9.529733 E; radius: 11.56km; RR=0.18; p= 0.0000000000000028), cluster 2 (coordinates: 45.567129 N, 8.990805 E; radius: 0km; RR=2.58; p= 0.0000000063), cluster 3 (coordinates: 45.505930 N, 8.759943 E; radius: 0km; RR=0.23; p=0.00029), cluster 4 (coordinates: 45.935558 N, 8.812758 E; radius: 15.57km; RR=1.68; p= 0.00089) and cluster 5 (coordinates: 45.903582 N, 8.635179 E; radius: 14.18km; RR=1.72; p= 0.0029). Cluster 4 and 5 partially overlapped (Fig. 10).

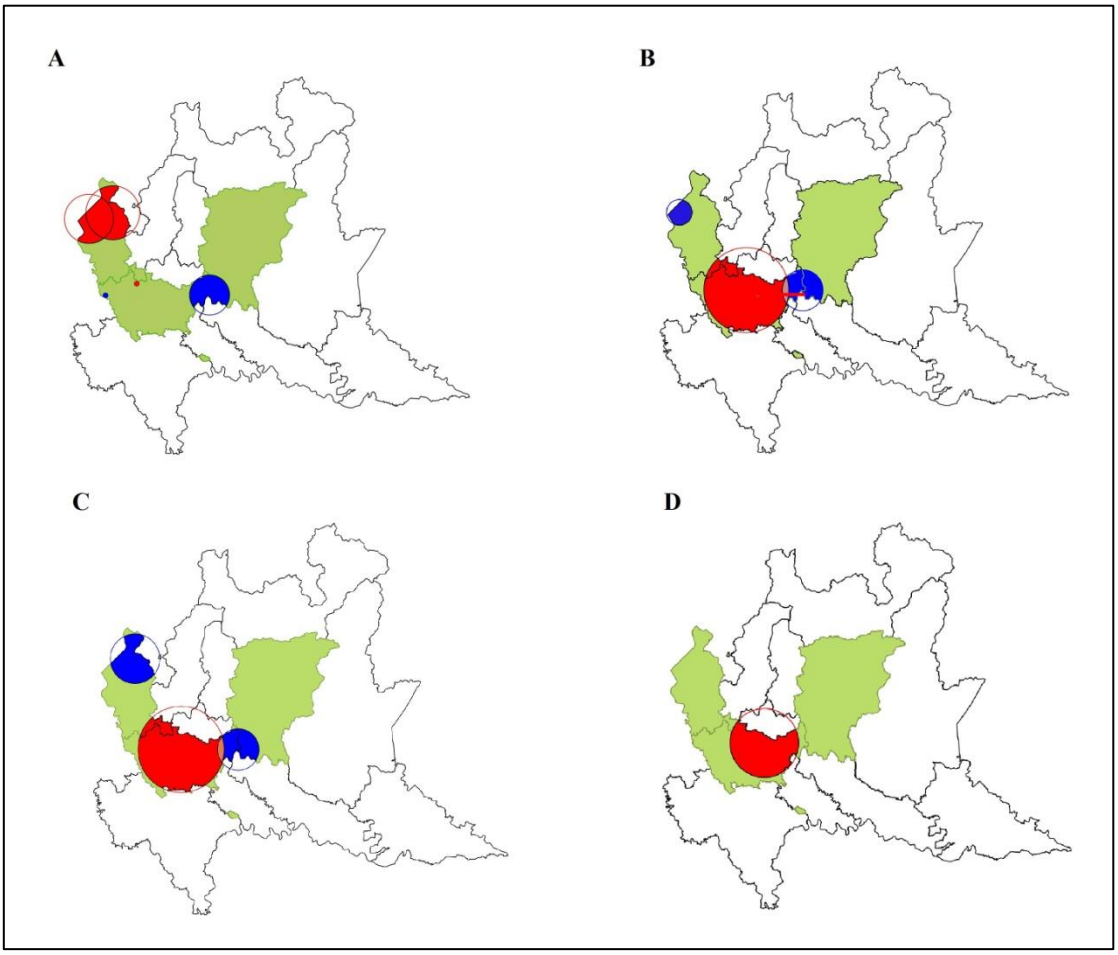

Fig. 10 -Clusters of *T. gondii* infection in goats generated by SaTScanTM analysis. Panel A: unadjusted clusters; panel B: age-adjusted clusters; panel C: size-adjusted clusters; panel D: management-adjusted clusters.

In the adjusted analysis for age, cluster 1 remained statistically significant at low risk, since it was not dependent on spatial distribution of considered variable. A new cluster overlapped cluster 2: it is a cluster at high risk not dependent on distribution of age. Another cluster of low risk was generated not dependent on spatial distribution of age (Fig. 10B). Cluster 1 remained statistically significant

also in the adjusted analysis for size, stating that it was not dependent on the distribution of the variable. The new clusters at high and low risk were calculated also in the adjusted analysis for size; they were not dependent neither on distribution of farm size (Fig. 10C). In the adjusted analysis on management, only a cluster at high risk appeared in the adjusted analysis for management, not dependent on distribution of management (Fig. 10D).

Therefore, considering results obtained from the adjusted analysis of the three variables considered, a cluster of high risk not depending on considered variables was calculated. The resulting overlapping area of high risk therefore apparently depended on geographical and environmental factors and not on the considered individual and managerial factors.

Climatic data were therefore considered and several variables entered in final model of the binary logistic regression analysis (Table 13). High temperature in winter and autumn resulted in a high risk of *T. gondii* infection (OR=7.655 and OR=8.675, respectively); on the contrary, with the increasing of temperature in spring and summer the risk of *T. gondii* infection decreased (OR=0.264 and OR=0.051, respectively). Rainfall in winter and spring (OR=1.308 and OR=1.246) resulted significant related to seropositivity to *T. gondii*, increasing the risk of infection with the increasing of rainfall. On the contrary, rainfall in summer and autumn resulted inversely related to *T. gondii* infection (OR=0.751 and OR=0.311). Interactions between temperature and rainfall resulted significant in winter, summer and autumn.

Table 13 - Risk factors associated with T. gondii infection in goats according to multivariate analysis.

Variable	Odds ratio	95 % CI	p-value
minimum temperature in winter	7.655	2.503-23.414	0.000
minimum temperature in spring	0.264	0.111-0.628	0.003
minimum temperature in summer	0.051	0.009-0.283	0.001
minimum temperature in autumn	8.675	2.240-33.592	0.002
rainfall in winter	1.308	1.155-1.482	0.000
rainfall in spring	1.246	1.092-1.422	0.001
rainfall in summer	0.715	0.554-0.923	0.010
rainfall in autumn	0.311	0.167-0.581	0.000
rainfall in winter × minimum temperature in winter	0.989	0.984-0.994	0.000
rainfall in summer × minimum temperature in summer	1.001	1.000-1.002	0.003
rainfall in autumn × minimum temperature in autumn	1.008	1.003-1.012	0.000

Considering now results obtained in the spatial analysis of data on sheep, four clusters were calculated: cluster 1 at low risk (coordinates: 45.841648 N, 8.637259 E; radius: 3.65km; RR=0.20; *p*=0.00031); cluster 2 at high risk (coordinates: 45.523788 N, 9.232348 E; radius: 0km; RR=2.30; *p*=0.00069), cluster 3 (coordinates: 45.508855 N, 9.122400 E; radius: 0km; RR=0.10; *p*=0.00076) and 4 (coordinates: 45.526072 N, 8.877204 E; radius: 0km; RR=1.83; *p*=0.049) at high risk (Fig. 11).

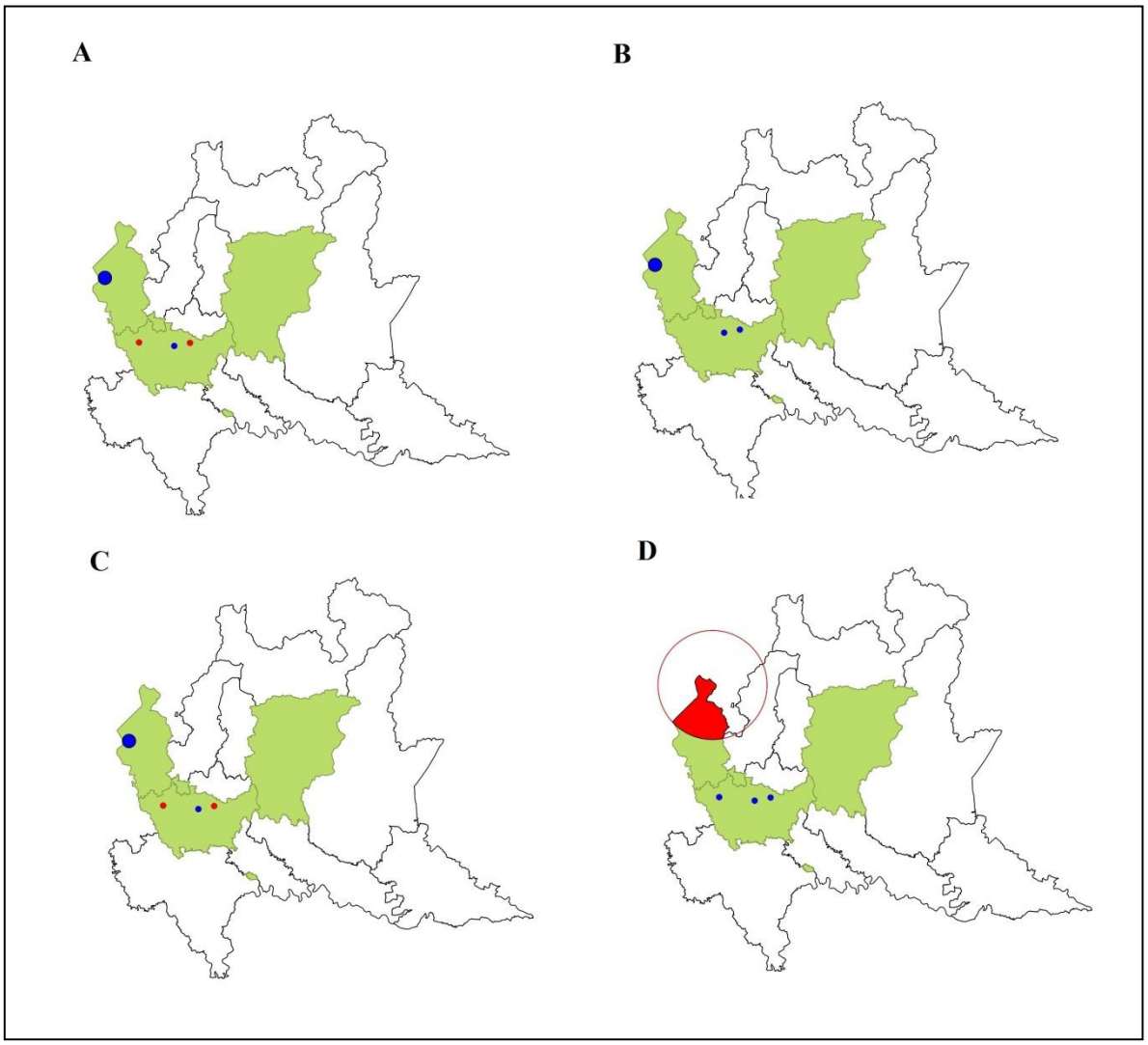

Fig. 11 - Clusters of *T. gondii* infection in sheep generated by SaTScanTM analysis. Panel A: unadjusted clusters; panel B: age-adjusted clusters; panel C: size-adjusted clusters; panel D: management-adjusted clusters.

In the adjusted analysis for age, cluster 1 and 3 remained statistically significant, stating that it was not dependent on the distribution of the variable. Cluster 2 became at low risk, stating that the distribution of the variable influenced the cluster in the unadjusted analysis. Cluster 4 disappeared since it was dependent on the distribution of the variable and not to geographical or environmental

factors. In the adjusted analysis for size, all cluster remained statistically significant, stating that all of them were not dependent on the distribution of the variable. In the adjusted analysis for management, cluster 1 disappeared and a new cluster was calculated (coordinates: 46.070704 N, 8.829239 E; radius: 29.456km; RR=0.18; *p*=0.000095): the new cluster is not due to spatial distribution of the variable and resulted at high risk; it comprised only farm with management M2 (semi-intensive). Cluster 3 remained statistically significant, stating that it was not dependent on the distribution of the variable. On the contrary, clusters 2 and 4 became at low risk, stating that the distribution of the variable influenced the calculation of the clusters in the unadjusted analysis.

Considering the whole results from the adjusted analysis for all considered variables, cluster 3 apparently was not depending on the spatial distribution of variables; it corresponded to a semi-extensive ovine farm with a *T. gondii* seroprevalence (P=5.2%) lower than other farms in the survey (overall P=59.3%).

Further, climatic data were considered. For sheep, the variables minimum temperature in winter and summer, and rainfall in winter were kept in the final model (Table 14). Temperature resulted highly significant, although the risk of *T. gondii* infection was differently related to the temperature in winter and summer: with the increasing of temperature, the risk increased in winter (OR=57.214) and decreased in summer (OR=0.100). Similarly, with the increasing of winter rainfall, the risk of infection increased (OR=1.462).

Table 14 - Risk factors associated with T. gondii infection in sheep according to multivariate analysis.

Variable	Category	Odds ratio	95 % CI	p-value
minimum temperature in winter	continuous variable	57.214	10.512-311.397	0.0001
minimum temperature in summer	continuous variable	0.100	0.039-0.255	0.0001
rainfall in winter	continuous variable	1.462	1.042-2.052	0.028

Statistically significant data in bold.

2.4. Spatial analysis of *N. caninum* infection: Results

Spatial analysis highlighted a heterogenic distribution of cases of infection, creating a cluster of infection both for sheep and goats.

Particularly, for goats a cluster of major infection was created in the province of Varese (coordinates: 45.935558 N, 8.812758 E; radius: 15.65 km; RR=5.08; p=0.0049) (Fig. 12).

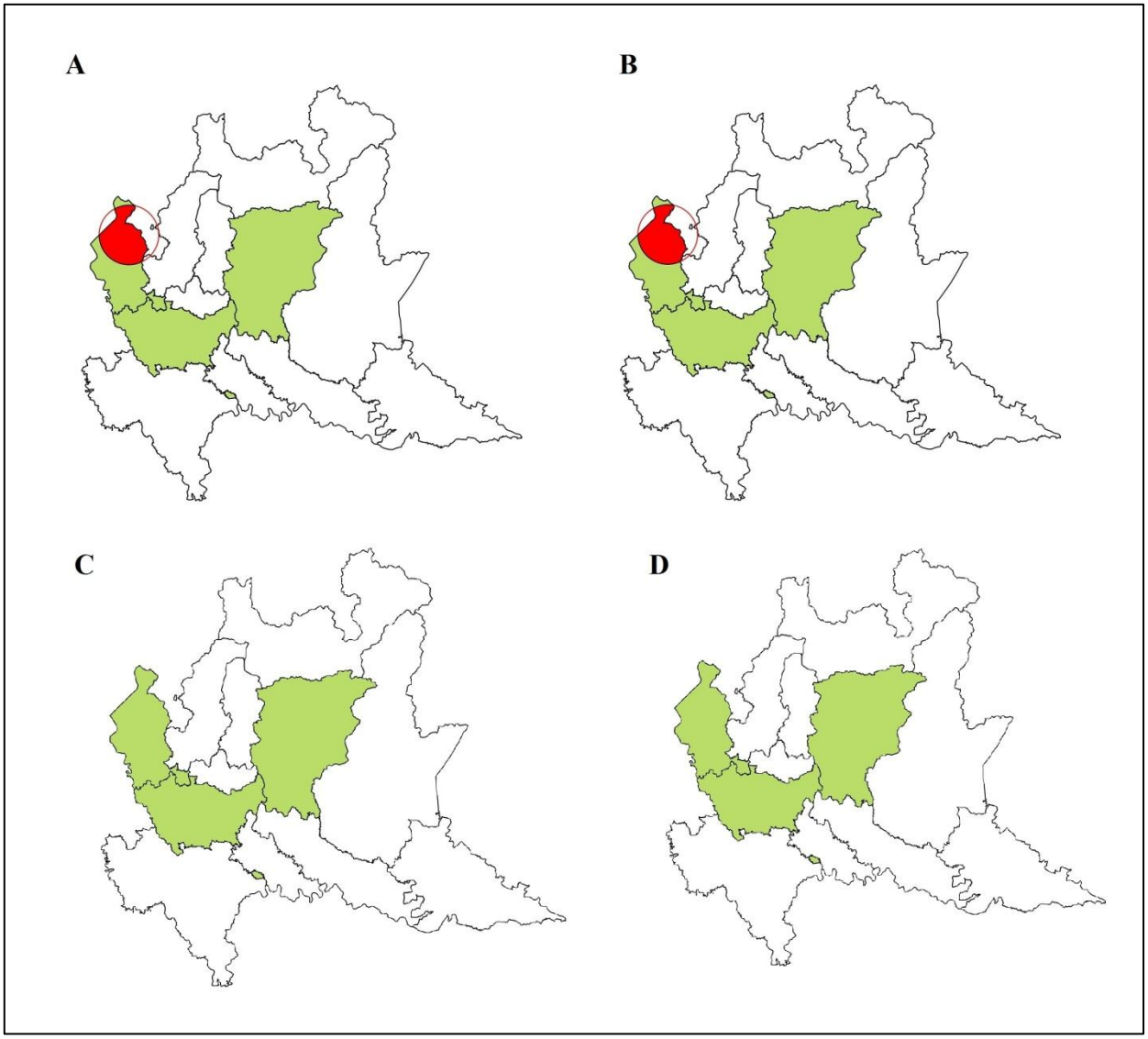

Fig. 12 - Clusters of *N. caninum* infection in goats generated by SaTScanTM analysis. Panel A: unadjusted clusters; panel B: age-adjusted clusters; panel C: size-adjusted clusters; panel D: management-adjusted clusters.

Also for sheep a cluster was created corresponding to an area in the province of Varese (coordinates: 45.526072 N, 8.877204 E; radius: 17.81 km; RR=0.24; p=0.0093) (Fig. 13).

Fig. 13 - Clusters of *N. caninum* infection in sheep generated by SaTScanTM analysis. Panel A: unadjusted clusters; panel B: age-adjusted clusters; panel C: size-adjusted clusters; panel D: management-adjusted clusters.

Considering the adjustment of the spatial distribution of samples related to the age, the same clusters remained statistically significant in the analysis of both species, stating that they were not dependent on the distribution of the variable.

On the contrary, both for sheep and goats, the managerial variables did not produce significant clusters of infection ($p>0.05$). Therefore, clustering of *N. caninum* infection for both species was dependent of the spatial distribution of the managerial variables and not of the individual age.

Further, climatic data were considered. For both sheep and goats, any of the "climatic" variable (minimum temperature and rainfall per season) were entered in the final model ($p>0.05$).

2.5. Discussion

The spatial analysis aimed to a deeper comprehension of the factors in the study area involved in the infections of *T. gondii* and *N. caninum* infections in small ruminants.

In goats, spatial analysis for *T. gondii* infection allowed to characterize an area of high risk of infection resulted from the overlap of clusters identified by the adjusted analysis of the considered factors: individual age, farm size and management (Fig. 10). This area of major risk did not thus depend on considered factors associated to animals or farm characteristics, but on geographical and environmental factors. This finding suggested investigating on factors that could be involved. Temperature and rainfall data were considered and entered in a model of binary logistic regression analysis; indeed, these climatic variables resulted in the statistical analysis as risk factors for *T. gondii* infection. Mild temperature resulted to be a major risk factor contributing to support the infection: higher temperature in winter months and lower in summer resulted very significant variables in the model. Rich rainfall in winter and spring and on the contrary minor rainfall in summer and autumn resulted as other risk factors associated to the infection. In goats, concluding, an area of major infection was identified; it could be hypothesized that ideal conditions for the maintenance of the infection existed, such as mild temperature and associated precipitation.

Regarding *T. gondii* infection in sheep, not all data were entered in the spatial analysis, since it was not possible to analyze from a spatial point of view data on transhumant herds; therefore data have to be interpreted as representative of only settled herd. Spatial analysis with SaTScan™ produced data not clearly interpretable: in the adjusted analysis for management, a cluster of high risk of infection was generated overlapping a cluster that in the unadjusted analysis of low risk of infection (Fig. 11). Probably, samples were not homogeneously distributed regarding managerial variables. Apparently, on data produced by SaTScan™ analysis, no geographical or environmental risk factors could be attributed to a major risk of *T. gondii* infection in this species. On the contrary, GIS and GLM analysis on climatic data (carried out on the whole sampled population) produced results similar to those obtained for goats: mild temperature in winter and summer, and rich winter rainfall were risk factors associated to the infection in sheep herds.

Regarding *T. gondii* infection in small ruminants, we may conclude that climatic variable, such as temperature and rainfall, are important risk factors enhancing the risk of infection. Particularly, temperature and rainfall in the more extreme months from a climatic viewpoint (winter and summer months) contributed to increase the risk of infection. An inference could be the major possibility for oocysts to survive in mild climate, as suggested by other Authors (Dumètre and Dardé 2003, Djokic et al. 2014). Environmental unsporulated oocysts are proven indeed to lose their capacity to

sporulate and thus to be infective after freezing (24 hours at -21°C or 7 days at -6°C) and heating (50°C for 10 min) (Dumètre and Dardé 2003).

Considering data on *N. caninum* infection, unadjusted spatial analysis revealed a cluster of infection at high and low risk of infection for goats and sheep, respectively (Fig. 12Fig. 13). These clusters could correspond to rural areas in which traditional farming of small ruminants is carried out with extensive or semi-extensive farms and it is characterized by small flocks. Instead, large intensive caprine farms and ovine transhumant flocks, presenting the lowest seroprevalence values for goats and sheep, are mainly located in the flatland and between Bergamo and Milano provinces. Subsequently, the adjusted spatial analysis showed that the cluster of major infection were actually due to the variable "management" both for sheep and goats and not to geographical or environmental features of the study area, confirming the importance of farm management as a risk factor for neosporosis.

Analysis on climatic data confirmed the absence of environmental, significant risk factors; it could be inferred that infection by *N. caninum* in small ruminants did not depend on environmental or geographical features but more on factors associated to farm management. On the contrary, individual age was not a risk factor neither in the statistical neither in the adjusted spatial analysis.

SaTScan™ and geospatial analysis in general are useful tools for the identification of clusters at high or low risk of infection. However, data should be interpreted considering factors concerning individuals and herds, since a not homogeneous distribution of cases (also due to territorial, zootechnical and economical characteristics) could lead to calculation of areas of infections not depending on geographical or environmental features but to structural characteristic of sampled herds. Moreover, climatic data should be considered in a geospatial analysis, since variables such as temperature and rainfall could contribute to maintain and spread the infection in the study areas.

Research line 2 –Diagnosis of *Toxoplasma gondii* infection in small ruminants by milk analysis and sanitary risks from ovine and caprine products

Introduction

T. gondii is recognized as one of the major abortigenic pathogen in small ruminant (Ortega-Mora et al. 2007). The epidemiologic survey carried out in Northern Italy (see Chapter 1) revealed high seroprevalence values both in ovine and caprine farms both at herd level (96.6% and 87.5%, respectively) and at individual level (41.7% and 59.3%, respectively).

The detection of *T. gondii* infection within a herd allows the planning of monitoring actions in order to reduce the percentage of seropositive animals (Bartels et al. 2007). However, in the perspective of a long-term and continuous monitoring, blood sampling and analysis of all animals or at least a representative part of animals in a farm is not economical affordable.

The possibility to use individual milk samples as alternative to blood samples has been investigated and validated for many pathogens; milk is easier to collect and sampling may be performed by un-specialized employees (Schares et al. 2004).

Therefore, in the following study we evaluated the possibility for a commercial ELISA (ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET, Montpellier) for the detection of antibodies anti-*T. gondii* to be used for milk analysis. A panel of individual goats' sera and milk pairs was used as reference in the validation of the test. The analysis on milk could be a useful tool for a fast and reliable diagnosis of *T. gondii* infection in the selected species.

Thereafter, in a farm previously found seropositive to *T. gondii*, the whole herd during the lactation period was monitored to deeper investigate the infection. Seroprevalence was above 60% and problems in fertility were reported, although never diagnosed. Among the abortigenic pathogens, *T. gondii* resulted indeed one of the most predominant when a laboratorial diagnosis is carried out (Ortega-Mora et al. 2007). The aim was to investigate on the variation of antibodies in sera and milk during the lactation. Few information was available on antibody kinetics of *T. gondii* infection in goats (Ferrer et al. 1997); similarly, Mesquita et al. (2013) monitored for *N. caninum* a caprine farm during gestation. A secondary aim of the study was to evaluate the fitter physiological period

to detect antibodies in milk and therefore to evaluate the better period to monitor *T. gondii* in a caprine herd through analysis on milk.

Next step of the research was indeed the use of tank bulk milk to screen herds for *T. gondii* infection. The analysis on tank bulk milk represents a reality in the screening of pathogens; as an example, ELISA on tank bulk milk have been validated and utilized for the diagnosis of ostertagiosis, fasciolosis, lung worm bronchitis and neosporosis. Although data have to be interpreted carefully since is representative of the whole herd but not of the individuals, analysis on bulk milk may be a valid diagnostic tool in a herd health monitoring or investigation program (Sekiya et al. 2013). Among Apicomplexa, mostly bovine neosporosis has been investigated through the analysis on tank bulk milk (Schares et al. 2004, Frossling et al. 2006, Varcasia et al. 2006, Bartels et al. 2007). Milk sampling is easier and cheaper to obtain in comparison to serum sampling. It is also less invasive for animals, with a improving of animal welfare and reduction of production losses due to stress. Moreover, with just one sample of tank bulk milk a representation of all milking animals may be obtained. Therefore, this study aimed to obtain epidemiological data on *T. gondii* infection in goats analyzing tank bulk milk with a previously validated commercial ELISA.

Toxoplasmosis in humans continues to be a public health problem worldwide; seroprevalence surveys on human infections in Europe showed indeed high values ranging from 35% and 60%. The consumption of raw or undercooked meat is considered one of the major risk of acquiring *Toxoplasma gondii* infection (EFSA 2007), especially in Europe, where it has been attributed 30–63% of infections (Cook et al. 2000, Tenter et al. 2000). Among the possibly infected meat, small ruminant products are a major source of toxoplasmosis, mostly in some countries and among certain ethnic groups the consumption of undercooked meat is a cultural and traditional habit (Kijlstra and Jongert 2008).

Since blood samples are difficult to obtain at slaughterhouses, we planned a serosurvey based on the detection of antibodies in meat juice. Different surveys have been carried out applying ELISAs in the diagnosis of toxoplasmosis using meat juice (Dubey et al. 2005, Berger-Schoch et al. 2011, Ranucci et al. 2012). The aim of the study was to obtain seroprevalence of *T. gondii* infection in ovine and caprine meats consumed in Italy. Both adult and young animals and both national and imported meats were sampled, in order to have a sampling representative of the meat eaten by Italian consumers. Although the detection of circulating antibodies does not necessary mean infectivity of meats, the detection of antibodies reflect the proportion of infected animals that therefore potentially represent a risk for consumers.

3. Validation of a commercial ELISA for the investigation of antibodies anti-*T. gondii* in milk

3.1. Materials and methods

Antibody analysis. A commercial ELISA kit (ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET, Montpellier) was utilized for milk analysis. Since the test is validated for ruminants only for serum and meat juice, a validation of the test for goat milk analysis was necessary.

For this proposal, in a dairy goat farm (see Chapter 4), blood and milk samples were collected. Blood samples, approximately 10 ml, were obtained from the jugular vein and collected in Vacutainer® tubes without anticoagulant agents. Contemporary, after disinfection of teats and using latex gloves, 10 ml of milk samples were collected from milking goats by manual milking and collected in sterile tubes. Specimens obtained were transported refrigerated to the laboratory in few hours. Once in laboratory, blood samples were centrifuged 2120g for 15 min, at room temperature; sera obtained were stored at -20 °C until analysis. Milk samples were processed according to (Petruzzelli et al. 2013) to eliminate the fatty components and the somatic cells of milk in order to avoid interference: 1 ml of milk was centrifuged at 13,000g for 30 min at room, and the milk-supernatant obtained was collected and transferred to another tube, then stored at -20°C until analysis.

Thirty goats' serum and milk-supernatant pairs were used for validation and tested at different dilutions to find the appropriate milk supernatant samples dilution. 19 positive and 11 negative goats' sera previously tested by IFAT were used as gold standard.

Sera were tested by ELISA at 1:10 dilution, according to the manufacturer instruction, whereas milk-supernatant samples were tested at 1:1, 1:2, 1:4 and 1:8 dilutions in the dilution buffer delivered with the ELISA kit. For each sample the resulted values were calculated applying the formula supplied in the kit:

$$S/P\% = (OD_{sample} - OD_{negative\ control} / OD_{positive\ control} - OD_{negative\ control}) \times 100.$$

Sera specimens with S/P% ≥50% were considered positive, whereas the cut-off value for milk-supernatant samples was calculated.

Statistical analysis. A linear regression analysis was conducted to state the optimal dilution for milk, using optical density (OD) results of sera and milk samples.

A non-parametric TG-ROC analysis was performed to determine the cut-offs values of S/P% at which milk-supernatant ELISA had the same sensibility and specificity relative the "gold standard". OD values of sera were used a reference.

Sensitivity and specificity of milk-supernatant ELISA were calculated as functions of the cut-off selected in the ELISA on sera, basing on a positive-negative classification. To evaluate the agreement between sera and milk analysis, Kappa statistics were calculated on sera-milk pairs.

3.2. Results

For the validation of the ELISA kit (ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET) for milk analysis, four experimental titrations were carried out. Using the O.D. values for sera-milk pairs, a linear regression analysis was performed. The 1:2 dilution showed the highest range of linear correlation (R^2=0.775) (Table 15, Fig. 14). Therefore, the 1:2 dilution was used for the further analysis.

Table 15 - Results on linear regression analysis obtained on goats' sera-milk pairs with ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET.

	Milk supernatant dilutions			
	1:1	**1:2**	**1:4**	**1:8**
R^2	0.648	0.775	0.761	0.742

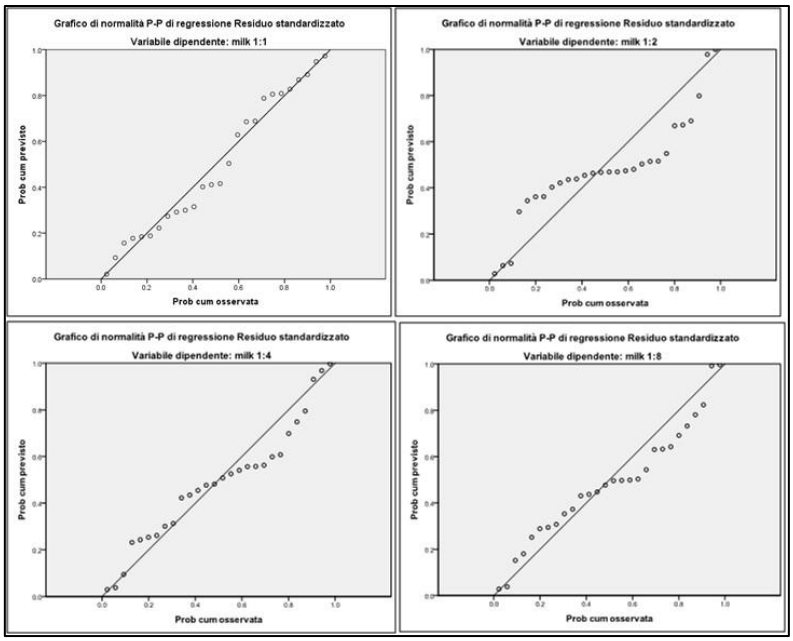

Fig. 14 - Linear regression on the results obtained on goats' sera-milk pairs with ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET. Sera samples were tested according to the manifacturer's instructions while milk samples at different dilutions.

To find the optimal cut-off for the examination of milk supernatant using the commercial ELISA, results on all 30 sera-milk pairs were analyzed by a non-parametric TG-ROC, considering sera

tested by IFAT the "gold standard". The analysis revealed an optimal cut-off for milk supernatant of S/P% = 25.5, at which sensibility and specificity were 95% and 93%, respectively (Fig. 15).

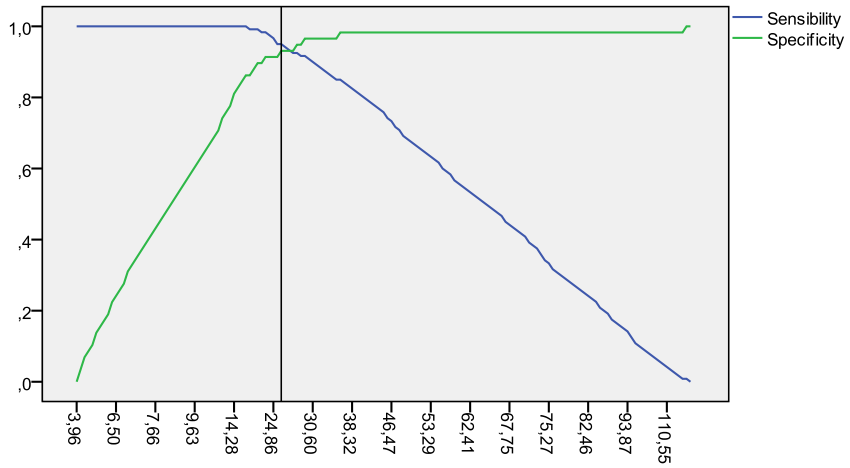

Fig. 15 - Results of a TG-ROC to determine the optimal cut-off for the examination of milk with ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET. An optimal cut-off was calculated on SP = 25.52, at which sensibility was 95% and specificity 93%.

Using 1:2 dilution and S/P%=25.5 as cut-off, 17 milk samples resulted positive out of 19 positive sera samples (89.4%); animals resulted negative in sera analysis were confirmed negative also by milk analysis (Table 16).

Table 16 - Results obtained from the comparison of ELISA in milk and sera in 30 goats.

		milk		
		negative	positive	overall
Sera	Negative	11	0	11
	Positive	2	17	19
	Overall	13	17	30

To state the agreement between sera and milk results, on these sera-milk pairs a Kappa statistic was performed and a 0.862 value was obtained (excellent agreement).

3.3. Discussion

The comparison between sera and milk results obtained by a commercial ELISA ID Screen® Toxoplasmosis Indirect Multi-Species (IDVET) showed a high agreement and a good linear correlation. An optimal dilution (1:2) was obtained and an appropriate cut-off (S/P%=25.5), with good sensitivity and specificity, was calculated; these parameters would be used for the subsequent analysis on milk. A good agreement (K=0.761) between results on sera and milk was obtained; thus, the analysis of milk samples with the commercial ELISA may be considered a valid alternative to testing sera. Collecting milk is easier and less expensive than collecting sera samples, as well as less stressing for the animals. It can be used as a valid tool for a first approach to the diagnosis of toxoplasmosis at farm and individual level (Schares et al. 2004).

Other serological tests have been developed and optimized in order to be applied on milk analysis. Generally, antibodies in milk may be used to measure the infection although it is to be considered that they appeared in milk later and at lower concentration in comparison to sera (Butler 1983).

(Sekiya et al. 2013) reviewed serological tests that have been performed for the research of parasites in bovine bulk milk. Particularly regarding Apicomplexa, ELISAs on milk were developed mostly to investigate on neosporosis in cattle (Schares et al. 2004, Varcasia et al. 2006, Byrem et al. 2012). Recently, an Iscom ELISA previously validated for cattle bulk milk for the detection of antibodies anti-*N. caninum* was evaluated when performed on individual milk samples of dairy sheep (Mula et al. 2012). Regarding toxoplasmosis, recently an IFAT was evaluated on ovine milk samples (Da Silva et al. 2014). On the basis on published results, a kappa statistic of 0.579 was calculated; the agreement between sera and milk increased when the antibody titer was higher than 1:64. When pooled sampled or tank bulk milk were analyzed, sensitivity increased: IFAT revealed a useful diagnostic tool to identify infected herds when applied at herd level. As observed by Bartels et al. (2007), the number of seropositive animals in herds (i.e. intra-herd seroprevalence) contributes to the antibodies level recorded by serological tools so differences in S/P% or antibody titer are to be taken into account. Comparing our results to those obtained by IFAT, a better agreement between sera and milk was found in ELISA (0.862) in comparison to IFAT (0.579). ELISA revealed to be more sensitive than IFAT; moreover, it is easier to perform on a large number of samples.

Concluding, ELISA on milk revealed a useful tool for the diagnosis of toxoplasmosis; testing milk samples could be an easy and valid first step in the detection of *T. gondii* infection in small ruminant breeding.

4. Case study of *T. gondii* infection in a naturally infected caprine herd: variation of antibody response in serum and milk during lactation

4.1. Materials and methods

Herd description. The study was carried out in a dairy goat farm in Varese province (Lombardy region, Northern Italy) housing 28 Alpine goats and two crossbreeds. This herd was selected among previously surveyed farms: it resulted indeed positive for *T. gondii* infection with an intra-herd prevalence of 61.2%. The farmer and the veterinarian were contacted and proposed for monitoring and investigation.

The farmer produced cheese from raw milk directly in farm; milk and products were then sold at local marketplaces. This kind of traditional farming is typical of caprine breeding in northern Italy and contributes to the safeguard of the economy of territories otherwise often abandoned. The veterinarian practitioner that followed the farm reported previous problems in fertility in eight animals: abortion at different stage of pregnancy, repeated heats, failed insemination. However, any diagnosis of abortion by laboratorial analysis was not ever carried out.

Animals were clinically and serologically monitored fortnightly for seven times within a period comprised from March and July 2013, corresponding to the peripartum period through the whole lactation for the majority of goats. Heats were indeed not synchronous in the group; therefore parturitions occurred at different times. The first two parturitions occurred in February; the major part of births occurred in March (16 goats), mainly in the first fortnight; five kids were born in April, two in May and the last one in June. Day of lactation was calculated for each goat taking the parturition as day 0. At sampling time, blood samples were collected from each animal and milk collected from milking goats.

Sample collection. Blood samples, approximately 10 ml, were obtained from the jugular vein and collected in Vacutainer® tubes without anticoagulant agents. Contemporary, after disinfection of teats and using latex gloves, 10 ml of milk samples were collected from milking goats by manual milking and collected in sterile tubes. Specimens obtained were transported refrigerated to the laboratory in few hours. Once in laboratory, blood samples were centrifuged 2120g for 15 min, at room temperature; sera obtained were stored at -20 °C until analysis. Milk samples were divided into two aliquots: the first one, for molecular analysis, was stored at-20°C until analysis. The second one, for serology, was processed according to (Petruzzelli et al. 2013) to eliminate the fatty components and the somatic cells of milk in order to avoid interference during the ELISA

processing: 1 ml of milk was centrifuged at 13,000g for 30 min at room, and the milk-supernatant obtained was collected and transferred to another tube, then stored at -20°C until analysis.

Antibody analysis. A commercial ELISA kit (ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET, Montpellier) was utilized for both sera and milk analysis.

Sera were tested by ELISA at 1:10 dilution, according to the manufacturer instruction, whereas milk-supernatant samples were tested at 1:2 dilution according to our results on validation of ELISA on milk analysis.

Positive and negative control sera provided with the kit were used as controls both for sera and milk-supernatant samples, but adding to each control 10µl of negative milk when used as controls for milk-supernatant samples. For each sample the resulted values were calculated applying the formula supplied in the kit:

S/P% = ODsample-ODnegative control / ODpositive control- ODnegative control) x100.

Sera specimens with S/P% ≥50% and milk-supernatant samples with S/P%≥25.5%were considered positive.

Statistical analysis. To evaluate the agreement between sera and milk analysis and between milk ELISA and PCR, Kappa statistics were calculated on all sera-milk pairs (n=150).To confirm the correspondence between sera and milk results, a Pearson's correlation analysis was carried out selecting sera-milk pairs according to day from parturition. Samples were divided into six categories corresponding to fifteen days each: 1° (0-15 days from parturition), 2° (16-30), 3° (31-45), 4° (46-60), 5°(61-75) and 6° (>76).

A mixed general linear model (GLM) for repeated measures was carried out to state the variation of antibodies in serum and milk during the lactation; two different models were created for data on sera and milk using ELISA S/P% results as continuous variable. Data were grouped into the same six categories previously described used corresponding to fifteen days each.

Further, a GLM was performed to determine factors that could be considered predictors of the presence of antibodies to *T. gondii* in serum and milk. Two models were created for serum and milk, respectively. When serum was used as dependent variable, milk was added as covariate variable and vice-versa. Other independent variables were entered the model: day of lactation (continuous variable, computed on the day of parturition) "age" (continuous, computed in months), and "problems in fertility" (dichotomous variable: presence/absence). In both GLMs, S/P% values were logarithmically transformed in order to normalize distribution. Statistical analysis was performed using SPSS (version 19.0; SPSS, Chicago, IL).

4.2. Results

To state the agreement between sera and milk results, on all sera-milk pairs a Kappa statistic was performed and a 0.873 value was obtained (excellent agreement).

The results on ELISA analysis both in sera and milk revealed a situation of *T. gondii* infection within the farm, with 63.3% of naturally infected goats (19 positive out of 30 animals). In Table 21, results from ELISA on sera and milk are resumed. Seronegative goats did not seroconvert during the whole survey period, with the exception of a goat (goat 16), that resulted seropositive only in two sampling (sampling II and VI) with S/P% values slightly above the cut-off (Table 21).

All seronegative goats were also negative to milk analysis; besides, no animals were positive to milk and negative to serum. The same occurred in positive animals, with positive animals in sera also positive in milk. We recorded a disagreement only in three sera-milk pairs: three positive sera samples (with low IgG values) had the correspondent milk samples negative, with values quite below the cut-off value.

To confirm the correspondence between sera and milk results, a Pearson's correlation analysis was carried out, showing a high agreement (0.815). The analysis was then performed selecting sera-milk pairs according to day from parturition: the best agreement was in the first fortnight since parturition (0.897) and then correlation decreases (Table 17).

Table 17 - Variation during the lactation in Pearson's correlation between sera and milk results.

	Days from parturition					
	0-15	16-30	31-45	46-60	61-75	>76
Pearson's chi-square	0.910	0.863	0.834	0.818	0.800	0.760

Indeed, our results indicated a different trend in antibodies level in sera and milk: S/P% values were high both in sera and milk in the first fortnight, then in the second fortnight (16-30 days from parturition) it increased even more in sera but decreased in milk. Seric globulins instead began to decrease a month after the beginning of lactation. The lowest values were registered in the fortnight comprised between the 46^{th} and 60^{th} day of lactation, and subsequently immunoglobulins increased again both in sera and milk at the end of lactation (Table 18).

Table 18 - ELISA S/P% results for sera and milk: mean and standard deviation variations during the lactation.

	ELISA S/P% sera		ELISA S/P% milk	
days from parturition	**mean**	**standard deviation**	**mean**	**standard deviation**
0-15	81.75	45.81	47.29	33.07
16-30	96.87	50.15	44.67	31.22
31-45	86.84	47.17	42.16	28.85
46-60	70.39	38.06	41.14	27.59
61-75	75.45	41.30	45.51	28.09
>76	87.86	48.23	50.99	33.25

In the GLM for repeated measures on sera data, we considered data on sera and milk as grouped in six categories based on day of lactation. Since the circularity assumption of the variance-covariance matrix was not satisfied, Huynh-Feldt corrected significance levels was used (Potvin et al. 1990). Difference among groups were statistically significant (p=0.002); particularly, differences was evaluated through pairs comparison and group 2 (16-30 days) resulted statistically significant when compared to all other groups (p<0.05). Moreover, differences were recorded between 3 *vs.* 4 and 4 *vs.* 6. A box-plot graphic resumes these results (Fig. 16).

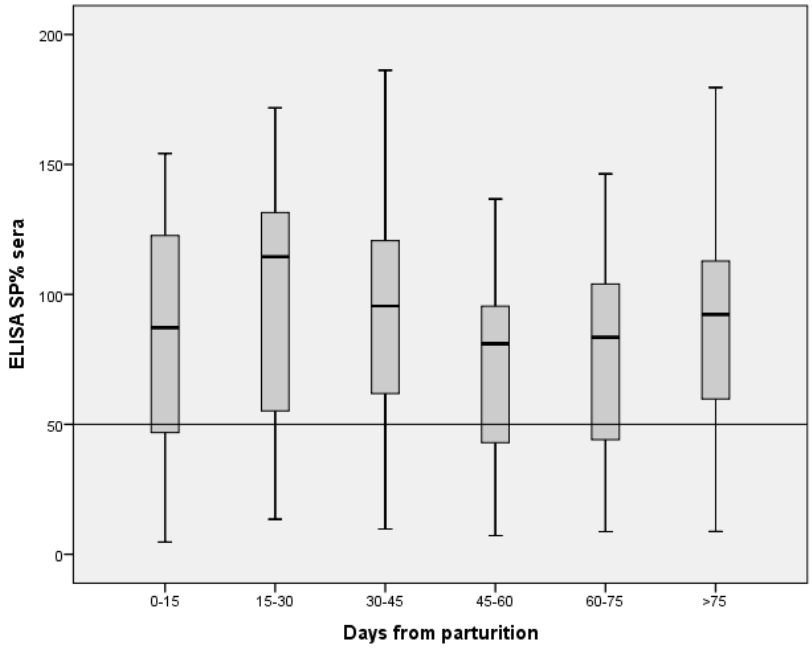

Fig. 16 - ELISA S/P% values on sera samples, during the lactation (calculated in days after parturition). Values above 50 are considered positive.

On the contrary, the same analysis on milk ELISA results did not produce any significant difference among group (Huynh-Feldt p>0.05). In Fig. 17, results on milk ELISA were resumed.

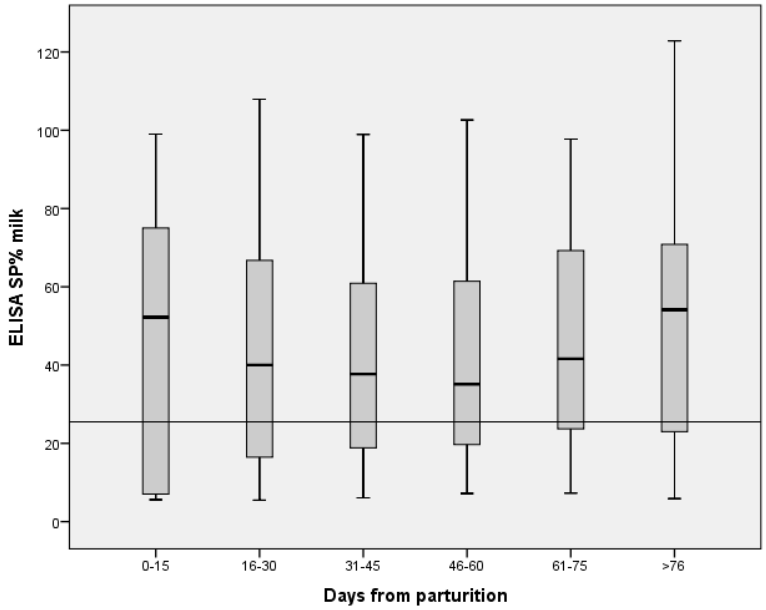

Fig. 17 - ELISA S/P% values on milk samples, during the lactation (calculated in days after parturition). Values above 25.5 are considered positive.

Further, GLMs were performed on sera and milk data, separately. In sera, antibodies level was influenced by age, days of lactation and antibodies level in milk (Table 19). Age was a risk factor positively related to *T. gondii* infection (OR=1.006; *p*=0.001), presenting adult animals higher values in comparison with young animals. The variable "lactation", computed in days from parturition, was negatively influent on seropositivity (OR=0.998; *p*=0.032). Finally, the level of antibodies in milk was a risk factor statistically significant (OR=2.429; *p*=0.0001). In the model, the variable "problem in fertility" was not statistically significant and was removed.

Table 19 - Results from GLM analysis on ELISA on goats' sera samples.

Variable	Category	Odds ratio	95% CI	p-value
Age	Continuous variable	1.006	1.002-1.009	**0.001**
Lactation (days from parturition)	Continuous variable	0.998	0.997-1.000	**0.032**
ELISA milk (Log S/P%)	Continuous variable	2.429	2.268-2.602	**0.0001**

Statistically significant variables are indicated by bold typing

In the GLM carried on milk results (Table 20), the variable lactation was significant but differently on sera analysis it resulted positively related to the infection (OR=1.002; *p*=0.044). ELISA results on sera had a significant effect for the presence of antibodies in milk (OR=2.455; *p*=0.0001). The variables age and "problems in fertility" were not statistically significant and were removed from the model.

Table 20 - Results from GLM analysis on ELISA on goats' milk samples.

Variable	Category	Odds ratio	95 % CI	p-value
Lactation (days from parturition)	Continuous variable	1.002	1.000-1.003	**0.044**
ELISA sera (Log S/P%)	Continuous variable	2.455	2.305-2.615	**0.0001**

Statistically significant variables are indicated by bold typing

Table 21 - ELISA results on sera and milk on 30 goats in seven sampling.

			1st sampling		2nd sampling		3rd sampling		4th sampling		5th sampling		6th sampling		7th sampling	
goat	**age**	**Reproductive disorders**	**serum**	**milk**	**serum**	**milk**	**serum**	**milk**	**serum**	**milk**	**serum**	**milk**	**serum**	**milk**	**serum**	**milk**
1	13	Abortion	-		-		-	-	-	-	-	-	-	-	-	-
2	13		-		-		-		-		-		-		-	
3	13		-		-		-		-	-	-	-	-	-	-	
4	37		+++		+++	+++	+++	++	++	+++	++	++	++	++		
5	37	Repeated heats	+++		+++		+++		++	+++	++	+++	+++	++	++	+++
6	37	Abortion	++		+++		++		+++		++		++		++	
7	25		-		-	-	-	-	-	-	-	-	-	-	-	-
8	87	Repeated heats	+		+++		++		++		++		+++	++	++	++
9	13		-		-		-		-		-		-			
10	13		-		-		-		-		-	-	-	-	-	-
11	87		++	++	+++	++	++	++	++	++	++	++	++	+	++	++
12	87		+++		+++	++	+++	+	++	+	++	++	+++	++	++	+++
13	61		++	+++	+++	++	+++	++	++	++	++	++	+++	++	++	++
14	74		++	++	+++	++	++	+	++	++	++	++	+++	++	++	+++
15	37		+	+	+++	+++	+++	+++	++	++	++	+++	+++	+++	++	+++
16	37	Repeated heats	-		+		-	+	-	-	-	-	+	-		-
17	25	Repeated heats	+++		+++		+++		++		++		+++		++	
18	25		-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	61	Abortion	+++		+++		+++		+++	+++	+++	+++	+++	+++	+++	++
20	50		+		+	-	+	+	+	+	+	+	+	+	+	+
21	50		++	++	++	+	++	+	++	+	++	+	+++	+++	++	+++
22	50		+++		+++	++	++	++	++	++	++	++	+++	++	++	++
23	50		+++		+++	+++	+++	++	++	+	++	++	+++	++	++	++
24	50		+		+++	+++	++	+++	++	+++	++	+++	+++	+++	++	+++
25	50		-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	61		++	++	++	+	++	+	++	+	++	+	++	+	+++	+
27	61	Abortion	++	++	++	+	+	+	+	+	+	+	++	+	+	+
28	61		-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	74		+++	++	+++	+	++	+	++	+	++	+	++	+	++	+
30	61		-	-	-	-	-	-	-	-	-	-	-	-	-	-

For sera samples: <50=negative (-); 50-80= "+"; 80-120="++"; >120="+++".

For milk samples: <25.5=negative ("-"); 25.5-50="+"; 50-80="++"; >80="+++". Blank cells when samples lack (i.e. goat not milking).

4.3. Discussion

In the study we analyzed IgG anti-*T. gondii* in sera and milk samples collected in a goat farm that presented previous and not diagnosed problems in fertility. Thirty female goats were sampled for seven times, during the whole lactation period. The survey allowed collect information about the dynamics in antibody anti-*T. gondii* levels both in sera and milk during the lactation.

The correlation between sera and milk was calculated considering the different phases of lactation; the best correlation value was obtained during the first fortnight since the parturition day. Indeed, the day of lactation is a strong predictor of antibody level both in sera and milk. According to our data, IgG level is high in the first two week after birth, then decreases and has a second peak at the end of lactation both in sera and milk, although in sera high concentrations of antibodies were maintained for a longer period until the 45th day post parturition.

Few data are published regarding the immunoglobulins level in goats' milk during the all lactation. (Ferrer et al. 1997) and (Levieux et al. 2002) reported high IgG values in the first three days and then the concentration of immunoglobulin decreases, and it correspond to the first 24-36 hours after birth in which intestine of newborns goats can adsorb immunoglobulins from milk (Mesquita et al. 2013). The second peak we registered at the end of lactation may correspond to a phenomenon registered in cattle: in cows, in fact, some studies showed a increasing at the end of lactation due to a decrease in milk yield and consequently a major milk protein and IgG concentrations (Schares et al. 2004, Chanlun et al. 2006).

Although in sera IgG appear earlier and in greater concentration than in milk (Sekiya et al. 2013), the curve of level of antibodies in milk appeared to reflect those in serum: therefore, the trend in milk may correspond to the trend of systemic immunoglobulin production. Peaks of IgG in the first fortnight of lactation could also due to the decrease of immune response associated to the stress of parturition; however, further studies are necessary to correctly explain this mechanism.

From a diagnostic point of view, it results more convenient to analyze milk samples during the first fortnight from the parturition to have a data the more correspondent to sera.

5. Evaluation of caprine tank bulk milk as diagnostic tools for *T. gondii* infection

5.1. Materials and methods

Milk sampling and processing. With the collaboration of the Regional Breeders Association of Lombardy (ARAL, http://www.aral.it), one hundred samples of tank bulk milk of dairy goats farms in Lombardy region (Northern Italy) were collected within the control quality program. Data on farm size (i.e. number of animals on farm at sampling time) and number of milking in a day were provided; moreover, location of farm was registered.

Tank bulk milk was processed to eliminate fatty components and somatic cells in order to avoid interference during the ELISA processing (Elvander et al. 1995, Varcasia et al. 2006): 1 ml of milk was centrifuged at 13,000g for 30 min at room (Petruzzelli et al. 2013), and the milk-supernatant obtained was collected and transferred to another tube, then stored at -20°C until analysis.

ELISA on milk. Samples were analyzed with a commercial ELISA kit (ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET, Montpellier) previously validated for analysis on milk. ELISA on milk-supernatant specimens was performed as previously described.

Statistical analysis. A multivariate binary logistic regression analysis was performed to determine factors that could be considered predictors of the presence of antibodies to *T. gondii* in caprine milk. Three independent variables were entered the model: province, farm size (n° of animals in farm), n° of milking in a day. The model was developed by backward elimination until all remaining variables were significant ($p<0.05$). Statistical analysis was performed using SPSS (version 19.0; SPSS, Chicago, IL).

5.2. Results

The analysis on milk revealed a presence of antibodies in 58 out of 100 samples (58%) of tank bulk milk, with a mean S/P% of 55.1 (standard deviation: 55; min-max: 0-165).

Farms located in the province of Milan showed the highest prevalence (80%), whereas those located in the province of Lecco showed the lowest value (33.3%) (Table 22). Considering the size of the sampled farms, the smallest ones, with less than 50 animals in farm at sampling time, presented lower seroprevalence values (53.3%) in comparison to bigger farm (61.1%) (Table 23). The number of milking in a day was also considered; farms that milked more than three times in a day showed

the lowest prevalence (28.6%) in comparison to those milking one (52.2%) or two (60.7%) times (Table 24).

Statistical analysis considered these three factors; all variable were removed from the model since did not results statistically significant (p>0.05).

Table 22 - ELISA results on caprine tank bulk milk according to province of provenience.

Province	n° of positive farm (examined)	Prevalence (%)	ELISA S/P%		
			mean	standard deviation	min-max
Bergamo	11 (16)	68.8	55.1	54	0-150.1
Brescia	12 (23)	52.2	48.9	49.6	0-144.5
Como	2 (5)	40.0	45.7	56.7	0-122.9
Milan	4 (5)	80.0	65.6	27.4	0-113.1
Lecco	1 (3)	33.3	22.1	27.4	59
Pavia	2 (5)	40.0	51.3	66.3	0-165
Sondrio	8 (14)	57.1	53.2	44.8	0-129.4
Varese	4 (6)	66.7	48.8	62.9	0-161.5

Table 23 - ELISA results on caprine tank bulk milk according to farm size.

Farm size	n° of positive farm (examined)	Prevalence (%)	ELISA S/P%		
			Mean	standard deviation	min-max
≤50 goats	24 (45)	53.3%	52.4	60.6	0-165
>50 goats	11 (18)	61.1%	54.5	50.8	0-138.3

Table 24 - ELISA results on caprine tank bulk milk according to number of milking in a day.

n° of milking / day	n° of positive farm (examined)	Prevalence (%)	ELISA S/P%		
			mean	standard deviation	min-max
1	12 (23)	52.2%	62.8	65.6	165
2	17 (28)	60.7%	49.4	51.1	138.3
>2	2 (7)	28.6%	22.2%	45.9	113.1

5.3. Discussion

In the present study, antibodies anti-*T. gondii* were detected in 58 out of 100 (58%) tank bulk milk samples of dairy goats using a previously validated commercial ELISA (see Chapter 3). This result confirms *T. gondii* infection as very common among dairy goats' farms in Lombardy, as previously demonstrated by the epidemiological survey carried out in small ruminants bred in the same region (see Chapter 1).

It is very interesting to compare results obtained in the epidemiological survey based on individual serum samples and in the survey based on tank bulk milk; nevertheless, it is necessary to pay close attention in the interpretation of such a comparison. Study area and sampled farms were different in the two studies. Further, two different materials were employed as analyzed samples: in milk, antibodies are approximately 30 times less concentrated and appeared later than in serum (Sekiya et al. 2013). Finally, different serological techniques were performed: IFAT for serum and ELISA for milk samples.

In the serosurvey a higher number of farms resulted positive: in fact, 28 farms out of 29 examined (96.6%) were scored as positive. A farm was considered positive if at least one animal was found positive. Considering results on tank bulk milk, any relation could be inferred from ELISA S/P% values, antibodies concentration in samples and the proportion of infected animals. In a previous study on bovine neosporosis, it was estimated that a farm resulted infected by tank bulk milk analysis when at least 10–15% of the lactating animals in the herd were infected with *N. caninum* (Björkman and Uggla 1999). For *T. gondii* infection any data was available in literature at this regards, although we may suppose a similar phenomenon. All results obtained in tank bulk milk are therefore to be considered possibly under-estimated.

In the study based on tank bulk milk, the highest prevalence value was registered in the province of Milan; on the contrary, in the serological survey farms located in the same province resulted less at risk of infection. However, few farms were sampled for each province, thus any conclusion regarding differences in prevalence by province may be inferred.

Further, in milk based study, big herds (>50 goats), usually corresponding to big intensive farms, resulted at higher risk in comparison to smaller ones, whereas in the serological survey the farm size resulted inversely related to the infection and small family-run farms resulted more at risk of infection.

A variable introduced in tank bulk milk based survey that was not considered in the serological survey was "milking frequency". When more than 2 milking in a day were performed, seroprevalence decreases. A recent study carried out in a autochthonous caprine bred in Canary Island demonstrated that milk components varied in concentration according to milking frequency.

Particularly, IgG concentration increased when milking frequency decreases from three to one milking per day (Hernández-Castellano et al. 2011). Indeed, in our survey farms performing only one milking in day showed the highest ELISA S/P% values; therefore, there were more probabilities to detect antibodies anti-*T. gondii* in one milking farms due to the high concentrations of immunoglobulins.

Concluding, high prevalence (58%) was recorded, as previously registered in the seroepidemiological survey. Analysis on tank bulk milk revealed a fast and economically affordable tool in the context of a health monitoring program; it is to be intended as a first screening to point out farms having seropositive to *T. gondii* in dairy goats. This kind of analysis allows performing epidemiological surveys on large scale and planning monitoring controls and selection within positive farms.

6. Serosurvey based on meat juice analysis on *T. gondii* infection in slaughtered small ruminants

6.1. Materials and methods

Sampling. During a period comprised from April 2013 and January 2014, 223 slaughtered sheep and goats were sampled from three slaughterhouses (two in Lombardy, northern Italy, and one in Rome, central Italy) and from a local retail meat store in Lombardy. The herds originated from several areas in Italy (Como and Varese in the North, Rome in Central Italy, Sardinia and Sicily in the South), Great Britain (Wales and Scotland), Greece and Romania to have a representative sample of meat consumed in Italy.

The number of sampled animals was proportioned according to age in order to picture a representative pattern of ovine and caprine meat consumed in Italy: indeed, in Italy 90.3% and 86.4% of slaughtered sheep and goats were <12 months old, respectively (ISTAT 2010). In our study, 87.4% of sampled animals were <12 months and the remaining 12.6% were adult animals (>1 year). Only adult sheep from Como were obtained; from Varese, lambs, kids and adult goats were sampled. From the other proveniences, it was possible to sample only lambs since no adult animals were imported at sampling time.

From each animal, hearth or diaphragm was obtained basing on convenience. The whole carcass was available of animals coming from Italy and hearth was removed and collected. From imported animals, carcasses arrived to the retail meat store already removed of internal organs (including hearths); only diaphragm was available and sampled. Hearts and diaphragms were collected in individual plastic boxes and kept refrigerated during transportation to laboratory.

Once in laboratory, specimens were maintained in plastic bag at -20°C overnight, subsequently defrosted at room temperature. The obtained meat juice was collected in tubes, centrifuged at 1000g for 10 minutes and then stored at -20°C until analysis.

Serology on meat juice. Meat juice of sheep and goats were tested for *T. gondii* antibodies with a commercial ELISA kit (ID Screen® Toxoplasmosis Indirect Multi-Species, IDVET, Montpellier). The test is validated for meat juice in ruminants and was thus performed according to manufacturer's instruction using 1:2 dilution. Positive and negative control sera provided with the kit were used as controls. For each sample the resulted values were calculated applying the formula supplied in the kit:

S/P% = (OD sample – OD negative control / OD positive control – OD negative control) × 100. Specimens with S/P ≥50% were considered positive.

Statistical analysis. A multivariate binary logistic regression analysis was performed to determine factors that could be considered predictors of the presence of antibodies to *T. gondii* in meat juice samples. Age (continuous variable, computed in months) and origin (categorical variable) were entered as independent variables. The model was developed by backward elimination until all remaining variables were significant ($p<0.05$). Statistical analysis was performed using SPSS (version 19.0; SPSS, Chicago, IL).

6.2. Results

Antibodies anti-*T. gondii* were found in 46 (20.6%) of 223 examined animals. However, slightly differences were noticed between goats and sheep and between young and adult animals. Young animals resulted less infected than adults, with an overall prevalence of 15.9% in <12months animals *vs.* 53.6% in adults. Also considering the two species separately, adult goats resulted more infected (53.6%) than kids (12.5%) and adult sheep (100%) more infected than lambs (16%). Although the overall prevalence resulted higher in sheep (19.9%) than in goats (25.9%) due to discrepancies in sampling, all sampled adult sheep presented antibodies anti-*T. gondii* vs. 31.6% of adult goats. In analogy, lambs resulted more infected (16%) than kids (12.5%) (Table 25).

Table 25 - ELISA results on meat juice of slaughtered small ruminants according to species and age.

	positive (examined)	P%	95% CI	ELISA S/P% mean	ELISA S/P% min-max
Overall	46/223	20.6	15.3-25.9	34.9	0-235.6
<12 m	31/195	15.9	10.7-21.0	29.7	0-233.1
>12 m	15/28	53.6	35.1-72	36.8	0-235.6
goat	7/27	25.9	9.4-42.4	26.6	0-133.3
<12 m	1/8	12.5	0-35.4	20.0	0-107.5
>12 m	6/19	31.6	10.7-52.5	26.6	0-133.3
sheep	39/196	19.9	14.3-25.5	34.9	0-235.6
<12 m	30/187	16.0	10.7-21.2	29.6	0-233.1
>12 m	9/9	100.0	70-100	74.3	0-235.6

Samples with ELISA S/P% ≥50 were considered positive.

Considering the provenience of sampled animals, both sheep and goats raised in Italy appeared to be more infected than imported animals, although it is to be considered that all imported animals in

the study are represented by lambs <12 months. Anyway, also taking into account young animals, lambs raised in Italy showed higher prevalence than those imported.

Table 26 - ELISA results on meat juice of slaughtered small ruminants according to provenience.

	Provenience	Species (age)	positive (examined)	P%	95% CI	ELISA S/P% mean	ELISA S/P% min-max
Imported	Romania	lambs	0 (27)	0.0	0-0.1	7.7	0-16.5
	Greece	lambs	1 (29)	3.4	0-10	23.3	0-70.6
	Scotland	lambs	1 (26)	3.8	0-11.5	10.3	0-52.4
	Wales	lambs	3 (29)	10.3	0-21.4	20.2	0-75.6
Italy	Varese	lambs	6 (6)	100.0	99.1-100	159.5	98.4-233.1
		adult goats	6 (19)	31.6	10.7-52.5	45.8	0-133.3
		Kids	1 (8)	12.5	0-35.4	20	0-107.6
	Como	adult sheep	6 (6)	100.0	99.1-100	182.7	113.7-235.6
	Rome	Lambs	11 (17)	64.7	41.9-87.4	98	0-208.2
	Sardinia	Lambs	5 (27)	18.5	3.8-33.1	26.8	0-99.5
	Sicily	Lambs	6 (29)	20.7	5.9-35.4	25.7	0-72.7

Considering risk factors analysis, age resulted statistically significant (p= 0.0001), increasing the risk of infection with the increasing of age. On the contrary, the variable "provenience" did not result statistically significant (p>0.05), maybe because of the low number of goats and adult sheep.

Table 27 - Risk factors associated to *T. gondii* infection in slaughtered goats and sheep according to multivariate analysis.

Variable	Category	No. examined	No. positive	Prevalence (%)	Odds ratio	95 % CI	p-value
Age	young (reference)	195	31	15.9	1		
	adult	28	15	53.6	1.045	1.024-1.065	**0.0001**

Statistically significant variables are indicated by bold typing

Therefore, a univariate analysis was performed only on "lambs" data, using "provenience" as independent variable: provenience resulted statistically significant (p=0.001). Pairwise comparisons statistical analysis was then performed. In particular, lambs from Rome and Varese resulted statistically different when compared to lambs from all other proveniences. Also lambs from Romania resulted statistically different to lambs of other provenience excepting Wales (p>0.05) (Table 28).

Table 28 - Pairwise comparison statistical analysis performed on data related to *T. gondii* infection in slaughtered lambs of different proveniences.

	Romania	Greece	Scotland	Wales	Varese	Rome	Sardinia
Greece	**0.034**						
Scotland	**0.038**	0.937					
Wales	0.067	0.296	0.339				
Varese	**0.0001**	**0.0001**	**0.0001**	**0.0001**			
Rome	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.002**		
Sardinia	**0.013**	0.066	0.08	0.383	**0.0001**	**0.001**	
Sicily	**0.006**	**0.037**	**0.045**	0.272	**0.0001**	**0.001**	0.838

Statistically significant variables are indicated in bold.

6.3. Discussion

The consumption of ovine and caprine meat products is considered a major way of human infection by *T. gondii* (Kijlstra and Jongert 2008). High seroprevalence values were registered in both sheep and goats worldwide, as confirmed also by the epidemiological serosurvey carried out in sheep and goats in Northern Italy (see Chapter 1). A survey on meat products based on the detection of antibodies in meat juice was planned to estimate the risk of infection for consumers represented by small ruminants' meats consumed in northern Italy.

Meat juice samples, more easy to collect at slaughter, have been proven as suitable for the screening of infections in different meat-producing animals destined to human consumption, including *T. gondii* (Halos et al. 2010, Berger-Schoch et al. 2011, Basso et al. 2013a, Astorga et al. 2014, Meemken et al. 2014). Different serological techniques have been employed for the detection of antibodies anti-*T. gondii* in meat juice samples; in the current study, a commercial ELISA validated for meat juice in ruminants was used. Moreover, other commercial ELISA kit or IFAT have been validated and used with excellent performance (Ranucci et al. 2012, Glor et al. 2013).

The overall prevalence recorded in the present study is moderate both for sheep and goats (19.9% and 25.9%, respectively). Although the finding of seropositivity did not surely represent a hazard for consumers, the proportion of seropositive animals gives an idea of the circulating infection among small ruminants' populations. According to other Authors (Halos et al. 2010, Berger-Schoch et al. 2011), an increasing in seroprevalence was registered with the increasing of age. Adult animals resulted indeed significantly more at risk of infection than kids and lambs. Differences in seroprevalence associated to age are in accordance with other serosurveys and may be explained by

the oral route of contamination, being adult animals more exposed to parasite in environment (Tenter et al. 2000, Halos et al. 2010, Spisak et al. 2010).

A risk factor associated to the infection in sheep was represented by the provenience of lambs: animals proceeding from two areas (Como and Varese provinces) resulted at more risk of infection in comparison to other areas. Generally, lambs imported from abroad showed lower seroprevalence values in comparison to Italian animals. (Halos et al. 2010) reported no significant differences between French and imported samples, but recorded differences within France, with a prevalence increasing from North-western region to Southern region.

In the present survey, high seroprevalence values of *T. gondii* infection both in ovine and caprine meats were registered, reflecting the proportion of infected animals potentially representing a source of human infection. Although the association between consumption of raw or undercooked meat and *Toxoplasma gondii* infection is demonstrated (Cook et al. 2000, EFSA 2007), at slaughter during meat inspection no test is performed to detect *T. gondii* in meats. Indeed, *T. gondii* tissue cysts are not detectable macroscopically and serological test are not mandatory. Nevertheless, meat juice samples are easy to collect also during meat inspection and could be performed during the standard procedures. The detection of antibodies in meat juice samples may represent an affordable tool to improve the surveillance and reporting system for *T. gondii*, contributing to monitor and control the infection in human population.

Research line 3 – Bovine besnoitiosis in cattle

7. Epidemiological survey of *Besnoitia besnoiti* infection in dairy and beef cattle bred in Northwestern Italy and Sardinia Island

7.1. Introduction

Besnoitia besnoiti is a protozoan parasite belonging to the group of cyst-forming coccidians (Apicomplexa, Sarcocystidae) closely related to *Toxoplasma gondii* and *Neospora caninum*. Similarly to other species belonging to the genus *Besnoitia* infecting ungulates, the life cycle of *B. besnoiti* is in part unknown: cattle represent the intermediate host, whereas the definitive host, if any, has not been yet identified. By analogy with other apicomplexan species, a carnivore, eventually the cat has been suggested as the definitive host (Basso et al. 2011, Olias et al. 2011). Hematophagous insects (*Glossina*, *Stomoxys* and Tabanids) are considered mechanical vectors (Lienard et al. 2013). Moreover, the possibility of infection through the close contact between animals and iatrogenically (with the repeated use of hypodermic needles), have been suggested as potential means of transmission (Basso et al. 2011, Alvarez-Garcia et al. 2013a). Animal trade and movement throughout countries has been identified as major risk factors for establishment of new bovine besnoitiosis foci in naive areas and countries (Alvarez-Garcia et al. 2013a). Furthermore, the role of wild animals as possible hosts of the parasite needs to be investigated; hitherto, only few cases of seropositivity in red deer and roe deer have been registered in Europe and any surveyed wild carnivores showed antibody against *B. besnoiti* (Millan et al. 2012, Gutierrez-Exposito et al. 2013). In Europe, bovine besnoitiosis is considered an emerging or re-emerging disease, with increasing geographical distribution and caseload. It is endemic in large areas in Spain, Portugal and France, while isolated outbreaks have been reported in Germany, Switzerland, Italy, Greece and Hungary (EFSA 2010, Alvarez-Garcia et al. 2013a, Alvarez-Garcia et al. 2014, Cortes et al. 2014, Gutierrez-Exposito et al. 2014, Hornok et al. 2014, Papadopoulos et al. 2014, Waap et al. 2014). In Italy, besides cases in imported cattle (Agosti et al. 1994, Biolatti et al. 2012), autochthonous outbreaks involving local breeds and/or native individuals of any breed have been reported in the central mainland part of the country (Agosti et al. 1994, Gollnick et al. 2010, Manuali et al. 2011, Mutinelli et al. 2011, Gentile et al. 2012). In contrast with the focal distribution of these outbreaks, two ELISA-based surveys revealed high seroprevalence values in Southern Italy (44.1% and 83% at

individual and farm level respectively) (Rinaldi et al. 2013) and Central Italy (29.4-52% and 94.6-100% at individual and farm level, respectively) (D'Avino et al. 2014) . Earlier, Gentile et al. (Gentile et al. 2012) taking into account the recurrence of a few besnoitiosis outbreaks and the high seroprevalence values in an infected farm hypothesized that besnoitiosis should be retained endemic in Italy.

According to EFSA (EFSA 2010), epidemiological surveys are recommended to monitor the spread of *B. besnoiti* in Europe and increase knowledge on its biology and associated risk factors. Several standardized diagnostic techniques have been developed such as ELISA, IFAT, MAT and Western Blot and a few of them have been validated recently in a European inter-laboratories trial. Particularly, in order to increase test performance and to obtain valuable epidemiological data the combination of ELISA with an *a posteriori* more specific technique have been recommended (Alvarez-Garcia et al. 2013a, Garcia-Lunar et al. 2013).

A cross-sectional survey was designed to investigate seroprevalence of *B. besnoiti* in so far little explored areas of Italy: northwestern Italy and Sardinia region, representing a huge variety of geographical and ecological features. Our main goal was contributing to the reliable representation of *B. besnoiti* distribution at the national scale, and the debate on tools for active surveillance of bovine besnoitiosis in Europe.

7.2. Materials and methods

Area description. The serosurvey was carried out in two separate areas: mainland northwestern Italy (including Lombardy, Piedmont and Liguria regions) and insular Italy (Sardinia region). Sampled areas were representative of a high variety of management systems and of differences in landscape and climate.

Northern regions in Italy host mainly intensive farms for calves' cycle and beef production (mean 800 animals per farm); the majority of farms from Lombardy and Piedmont are mainly located in the flatland of Po valley, in which the continental climate is characterized by very cold winter and hot-moist summer. The overall cattle population is of 1,484,000 and 815,000 in Lombardy and Piedmont, respectively and the purchase of spare breeding animals is mainly from abroad (292,593 in Lombardy and 226,147 in Piedmont representing 22.1% and 17.1%, respectively, of imported cattle in Italy in 2010), particularly from France.

Liguria, instead, is a narrow region bordered on one side by the Alps and Apennine mountains and on the other by the Mediterranean Sea; thanks to these geographical features, climate is quite mild

all year round. In this region, farms are smaller (mean 20 animals per farm) and located mostly in the central western area; beef breeding is more represented than dairy one for an overall of 14175 cattle and only 112 animals were imported from abroad. Sardinia is 20.000 km^2 island located in the Mediterranean Sea and few exchanges of animals with the continental regions characterize its farming activity; only 385 cattle were imported in the year 2010 from foreign countries. The number of bred cattle is very low, amounting to 251,000 heads. Data were obtained from ISTAT (ISTAT 2010).

Study population and sample collection. A cross-sectional study was carried out using the individual animal as the sampling unit. Farms in the study were stratified for productive category (dairy and beef) and then randomly selected from those included in the national plan for the control of bovine brucellosis. Sampling stratification were performed on the basis of administrative boundaries; therefore a minimum sample size for each sampled region was determined by using the program Winepiscope 2.0 (http://www.clive.ed.ac.uk/winepiscope/0) to exclude (if all samples are negative) a *B. besnoiti* seroprevalence ≤50% within the animals in the sampled herds at a confidence level of 95% and an error margin of 5%. Data on animal amounts were obtained by ISTAT (ISTAT 2010).

Within each selected herd, animals over 12 months were sampled by systematic random selection, proportionally to the number of animals present in the farm (mean 25; min-max: 15-75). All animals were sampled if the farm had less than 15 animals. Both dairy and beef farms were selected and included in the survey. On the whole, 3140 bovine blood samples from 126 farms (79 from Lombardy, 12 from Piedmont, 15 from Liguria and 20 from Sardinia) were collected between October 2012 and May 2013 by local veterinarians in conjunction with the sampling for the regional sanitary controls. Different breeds were sampled: Holstein Friesian and Piedmontese breeds were the most consistent, followed by other cosmopolitan (i.e. Charolaise and Limousine) and local breed (i.e. Italian Brown, Bruno-Sarda and Grey Alpine). GPS (global positioning system) coordinates of each farm were gathered to map its location. No signs of besnoitiosis or other clinical signs were signaled by veterinarians in sampled hosts. At sampling time, individual data on each sampled animal (gender, age, and breed) and on farm management (dairy or beef farm, and origin of animals: born in farm, bought in another Italian farm or abroad) were recorded.

Blood samples were collected from jugular or tail vein, conserved in tubes without anticoagulant agents and transported to the laboratory in few hours, then centrifuged (15 min, 2120 g). Sera were stored at -20 °C until analyzed.

Serology. Serum samples were analyzed for antibodies against *B. besnoiti* by an in-house ELISA standardized at the Animal Health Department (SALUVET) of the Complutense University of

Madrid (Fernandez-Garcia et al. 2010). To confirm the results, the sera tested positive in ELISA were later analyzed by Western Blot (WB). The ELISA and WB used in the present survey showed a sensitivity of 97.3% and 98.1% and a specificity of 94.6% and 97.7%, respectively (Garcia-Lunar et al. 2013). As control for both tests, positive and negative sera samples previously tested by IFAT and Western Blot were used (Fernandez-Garcia et al. 2010). Further, a IFAT a was employed on a panel of sera resulted positive to ELISA (Fernandez-Garcia et al. 2009).

ELISA. Sera were analyzed through a standardized in house ELISA as previously described (Fernandez-Garcia et al. 2010). Briefly, 100 µl of coating buffer (0.1M carbonate-bicarbonate, pH 9.6) containing *B. besnoiti* soluble extract (0.2 µg) was added to each well of a polystyrene microtitre plate (Immuno Plate Maxisorp; Nunc) and incubated overnight at 4ºC, then washed with Phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS-T) and finally blocked with PBST containing 10% horse serum. Wells were washed and incubated with 100µl of bovine sera diluted 1:100 in blocking solution, for one hour at 37ºC, then washed again. 100µl of a monoclonal anti-bovine IgG1/IgG2 peroxidase-conjugated (LSI laboratories) at 1/6000 dilution in PBS-T was added and incubated for one hour at 37ºC. After washing, 100µl of 2,2′-Azino-bis (3-Ethylbenzthiazoline- 6-sulfonic acid) substrate (Sigma) was added and incubated one hour at room temperature in the absence of light. After 20 minutes, the reaction was stopped by adding 100µl of 0.3M oxalic acid. Absorbance was measured as optical density (OD) values at 405nm using a microplate reader (Multiscan RC 6.0; Labsystems).Samples were analyzed in duplicate, and the mean value of the OD was converted into a relative index per cent (RIPC) by employing the following formula:

RIPC = (OD sample - OD-negative control)/(OD-positive control - OD-negative control) × 100. Samples with an RIPC ≥ 9.7 were considered positive.

Western Blot. Western Blot (WB) was performed under non-reducing conditions according to Fernandez-Garcia et al. (Fernandez-Garcia et al. 2009). Samples containing 2×10^7 *B. besnoiti* tachyzoites were deterged-disrupted at 95°C for 5 min with Laemmli buffer (Laemmli 1970), sonicated in an ultrasonic bath at 15°C for 15 min and then heated for 5 min at 95°C prior to use. Electrophoresis was performed in 15% polyacrylamide-DATD minigels and then transferred to a nitrocellulose membrane (Mini Trans-Blot Cell, Bio-Rad Laboratories, CA, USA). Precision Plus Kaleidoscope weight standards (Bio-Rad Laboratories, CA, USA) were subjected to electrophoresis to estimate the apparent molecular weights of the different antigens recognized by sera. Membranes were washed in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBS-T), and then incubated overnight in blocking buffer (TBS-T, containing 3% (w/v) bovine seroalbumin) (Roche Molecular, Biochemical, Mannheim, Germany). After washing in TBS-T, membranes were incubated with

sampled sera diluted 1/20 in blocking buffer for 1 h at 37°C, washed again and exposed to a monoclonal anti-bovine IgG1/IgG2 peroxidase-conjugated (LSI laboratories) (1:600) conjugated with peroxidase (LSI laboratories) and incubated for 1 h at 37°C, rewashed and finally developed using 4-chloro-1-naphtol (Bio-Rad Laboratories) as substrate. Images from the membranes were obtained using a GS-800 Scanner (Bio-Rad Laboratories, CA, USA) and analyzed with Quantity One1 quantification software v. 4.0 (Bio-Rad Laboratories, CA, USA). Samples were considered positive if presented at least three bands in at least two of the following areas: area I (72.5, 58.9 and 51.4 kDa), area II (38.7, 31.8 and 28.5 kDa) and area III (23.6, 19.1, 17.4 and 14.5 kDa).

IFAT. A panel of 61 sera, including nine sera confirmed positive to WB, resulted positive to ELISA was processed by IFAT with cut-off titre of 1:200 as described by Fernandez-Garcia (2009) (Fernandez-Garcia et al. 2009). Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) for IFAT were calculated using WB results as gold standard. Further, agreement between IFAT and WB was verified with Youden's test by the program Winepiscope 2.0 (http://www.clive.ed.ac.uk/winepiscope/0).

Data analysis. Apparent (AP) and true prevalence (TP) were calculated basing on ELISA and Western Blot results, both at individual and herd level (Thrusfield 1995). A farm was considered positive if at least one seropositive animal was found. A multivariate binary logistic regression analysis was performed on WB results to determine factors that could be considered predictors of seropositivity. Both individual and farm data were included in the analysis as independent variables: breed, origin (born in farm, born in another Italian farm, imported from abroad), age, region, production (dairy or beef). Gender was not included because of the numerical disproportion between males and females. Statistical analysis was performed with SPSS (version 21.0; SPSS, Chicago, IL).

7.3. Results

In ELISA, anti-*Besnoitia* antibodies were revealed in 712 out of 3140 samples (AP=22.68%; TP=18.8%). The RIPC values showed a high variability with most of the samples (66.9%) presenting low values comprised between 9.7% (i.e. cut-off value) and 20%, 26.3% moderate values, 3.7% moderate-high values whereas very few animals (3.4%) had higher RIPC values (>80) (Fig. 18).

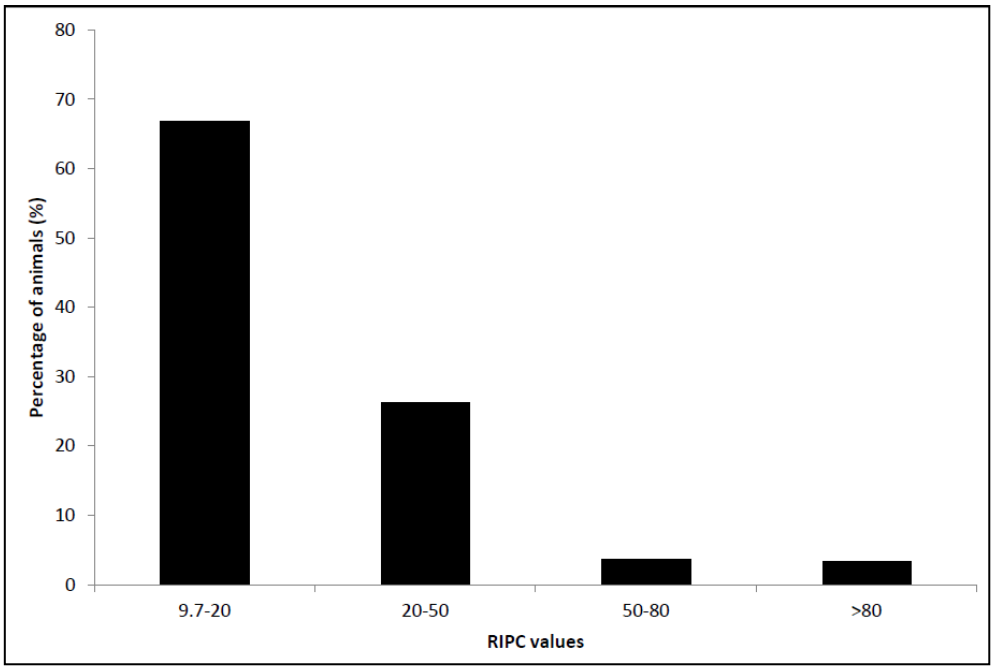

Fig. 18 - RICP values distribution in 712 cattle resulted positive to ELISA.

Of the positives cattle, 127 were beef cattle (AP=10.59%; TP=5.65) and 585 were dairy cattle (AP=30.13%; TP=26.91%). Overall, 109 farms (AP=86.5%; TP=88.25%) housed at least one ELISA seroreactor (Table 29). In particular, two dairy breeds, Italian Brown and Holstein Friesian, presented higher seroprevalence (27% and 38%, respectively) in comparison to the other considered breeds, which prevalence values ranged from 9% to 12%. Similarly, samples from dairy cattle seroreacted more than those from beef ones (33% and 10%, respectively). Considering the geographic regions, Lombardy presented the highest number of seropositive animals (28%) differently from Piedmont (10%), Liguria (13%) and Sardinia (12%).

The WB confirmed the presence of anti-*Besnoitia* antibodies in a minority of ELISA seroreactors; only ten cattle and five farms with AP of 0.31% and 3.97% at the individual and farm level, respectively were found positives with WB. For both levels, the TP was calculated at 0%. Samples confirmed positive by WB presented ELISA RIPC values ranging from 50.24 to 202.81 (Table 30).

Table 29 - Diagnostic of *Besnoitia besnoiti* infection in cattle by serological analysis (ELISA and WB).

	Production category (N° samples)	Serology Test	N° positive	AP	95% CI	TP	95% CI
Individual level	Overall (3140)	ELISA	712	22.68	21.22-24.14	18.8	17.45-20.14
		WB	10	0.31	0-0.72	0	0-0
	Dairy (1941)	ELISA	585	30.13	28.09-32.17	26.91	25.04-28.79
		WB	2	0.10	0-0.24	0	0-0
	Beef (1199)	ELISA	127	10.59	8.85-12.33	5.65	4.04-7.24
		WB	8	0.67	0.21-1.13	0	0-0
Farm level	Overall (126)	ELISA	109	86.5	80.53-92.47	88.25	82.74-93.73
		WB	5	3.97	0.56-7.38	1.74	0-5.00
	Dairy (77)	ELISA	77	100	95.32-100	100	95.32-100
		WB	2	2.59	0-6.14	0.3	0-3.7
	Beef (49)	ELISA	34	69.38	56.47-82.29	69.62	57.75-81.47
		WB	3	6.12	0-12.83	3.99	0-10.41

Particularly, out of WB positive animals 5 showed ELISA RIPC values of 50-80 and 5 >80. Cattle serum recognized all immnunodominant antigens described in each antigenic area (Fig. 19).

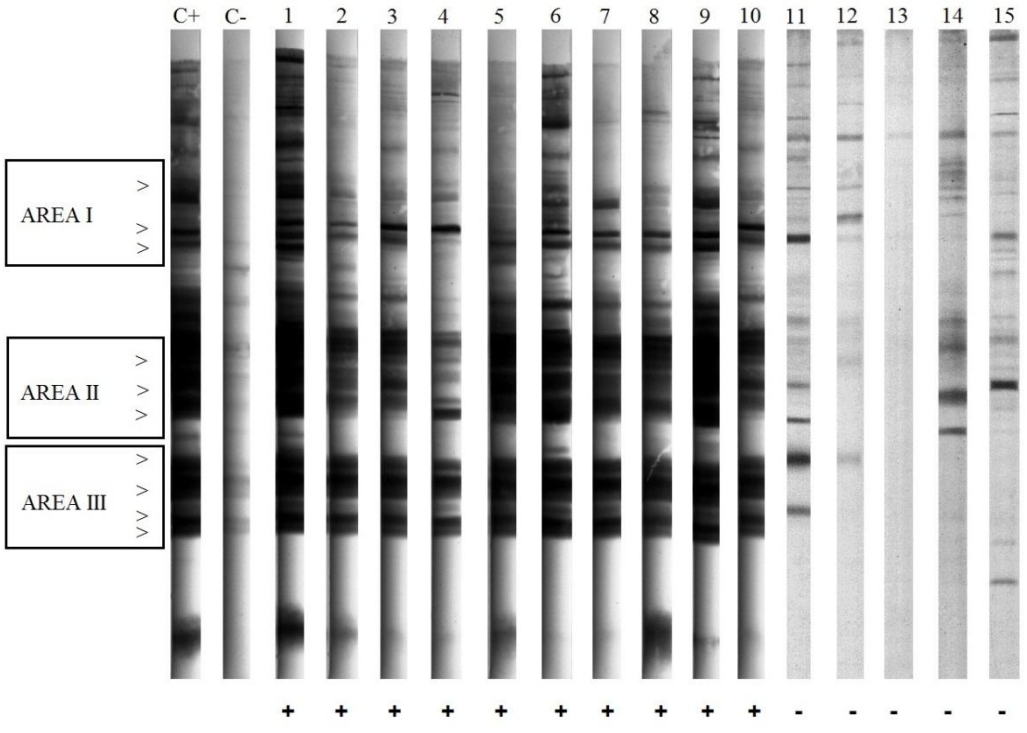

Fig. 19 - Pattern of recognition of *B. besnoiti* tachyzoite antigens by sera from naturally infected cattle by WB. Antigenic bands in the three main antigenic areas are indicated by arrows.

Risk factors analysis on data obtained by WB produced a non-fitting model resulting any of the considered independent variables not significant (P>0.05). Data on animals and farms testing positive in both ELISA and WB were summarized in Table 30, while the location of all sampled farms was represented in Fig. 20.All positive farms were located in Lombardy. Of the ten positive cattle, three were imported from France, five were born in the same farm where sampling was carried out and two were born in other farms in Italy. Five of the seroreactors belonged to the same farm A, a beef cow/calf operation housing about 700 Limousine adult cattle. Weaning and sale of calves occurs at 6-7 months. Natural mating is practiced and service bulls are mostly imported from France. Farm B is a lairage where cattle of different origin (Italy and a range of European countries) are rested on the way to domestic market. Farm C and D are located quite near the Apennine Mountains and particularly farm C is close to a beef cattle farm. They are dairy farms with intensive system housing approximately 400 and 200 Holstein Friesians, respectively; in both farms, artificial insemination (A.I.) is regularly practiced. Finally, farm E is another beef cow/calf operation housing about one hundred crossbreds. Cattle from A, B and E farms lived in paddocks, whereas cattle from C and D are housed in cubicles. Hygienic sanitary condition and animal welfare are very high in all of these farms.

Table 30 - Data on cattle resulted positives both to ELISA and Western Blot.

Farm	Region	Geographic Coordinates	Altitude (m)	Cattle n°	Breed	Gender	Age (months)	Production	Origin*	Time in the farm (months)	ELISA (RICP)§	WB
A	Lombardy	45°8'51"36 N; 09°51'20"16 E	41	1	Limousine	Female	159	Beef	I	135	130.81	+
				2	Limousine	Female	157	Beef	I	137	98.70	+
				3	Limousine	Female	157	Beef	I	137	191.88	+
				4	Limousine	Female	95	Beef	BF	95	80.61	+
				5	Limousine	Female	126	Beef	BI	118	55.50	+
B	Lombardy	45° 6'22.93"N; 9°17'1.10"E	62	6	crossbreed	Female	20	Beef	BF	20	73.16	+
				7	Limousine	Female	17	Beef	BI	16	68.53	+
C	Lombardy	45°14'55.77"N; 9°37'22.10"E	64	8	Holstein Friesian	Female	38	Dairy	BF	38	50.24	+
D	Lombardy	45°11'15.10"N; 9°44'8.93"E	59	9	Holstein Friesian	Female	98	Dairy	BF	98	60.40	+
E	Lombardy	45°6'51.96"N; 8°51'54.82"E	89	10	crossbreed	Female	14	Beef	BF	14	202.81	+

* I= imported; BF= born in the farm; BI= born in another Italian farm; §= cut-off>9.7

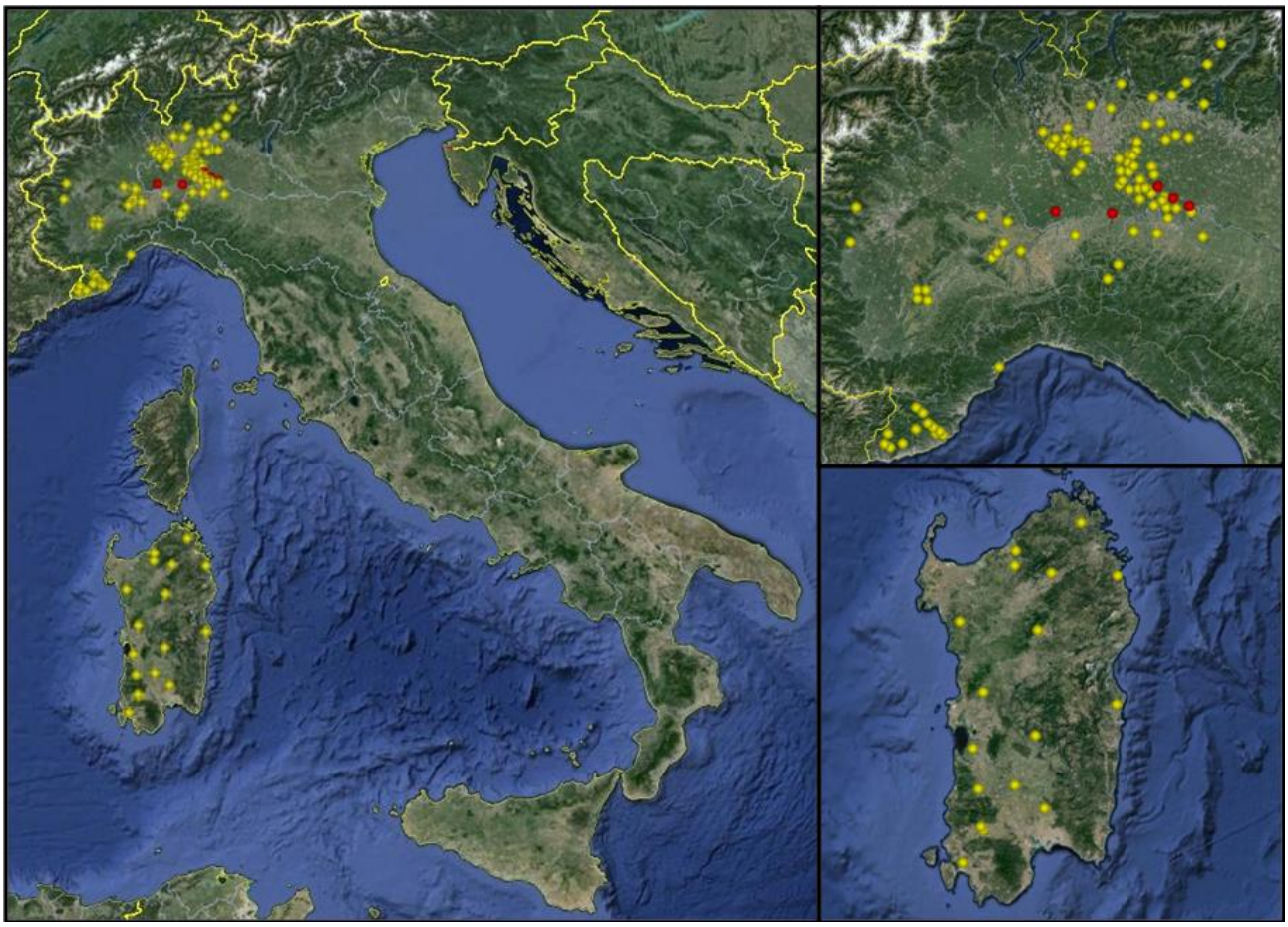

Fig. 20 - Distribution of farms of tested sera in the two samples areas: mainland northwestern Italy (including Lombardy, Piedmont and Liguria regions) and insular Italy (Sardinia region). Positive farms are in red.

In the IFAT, only eight sera (8/61, 15%) resulted positive at the threshold value of a dilution of 1:200. IFAT scored positive only four samples out of 9 positive to WB (Table 31). The IFAT performances were evaluated using WB results as gold standard; the test showed sensitivity of 44.4% (95%CI: 11.9-76.9%), specificity of 92.3% (95%CI: 85.1-99.5%), PPV of 50% (95%CI: 15.3-84.6%) and NPV of 90.5% (95%CI: 82.7-98.4%). The agreement between IFAT and WB resulted low (0.367).

Table 31 - Data on analysis with IFAT and WB on a panel of 61 sera resulted positives in ELISA.

	WB anti-*B. besnoiti*		
IFAT anti-*B. besnoiti*	WB +	WB -	Total
IFAT +	4	4	8
IFAT -	5	48	53
Total	9	52	61

7.4. Discussion

In recent years, EFSA recognized bovine besnoitiosis as an emerging or re-emerging infection in Europe (EFSA 2010). Endemic BB areas have been recognized in Spain, Portugal and France, however only isolated cases/outbreaks have been signaled in central and eastern European countries; in a few cases the infected animals were imported from France (Schares et al. 2009, Basso et al. 2013b, Hornok et al. 2014).In Italy, the first cases of BB were diagnosed two decades ago in imported beef cattle (Agosti et al. 1994), but reports of in autochthonous breeds date back to recent years (Gollnick et al. 2010, Manuali et al. 2011, Mutinelli et al. 2011).

The present serosurvey was designed to investigate the diffusion of *B. besnoiti* among beef and dairy cattle in little explored regions in mainland and insular Italy (Sardinia). Sampled farms were deemed representative of different geographical, ecological and management scenarios. Considering our data, BB seemed to be limited to sporadic and independent foci of infection in Lombardy region. In other considered region belonging to northwestern Italy, Piedmont and Liguria, any of sampled sera showed reaction in WB to *B. besnoiti*. Cases of BB were previously registered in Piedmont (Agosti et al. 1994, Biolatti et al. 2012), therefore we cannot exclude the presence of foci of infection in areas or herds not included in the survey. Of particular interest is the absence of BB in Sardinia, probably thanks to its geographical features and to the limited exchange for the purchase of spare breeding animals that contribute to prevent the spread of infections from the continental areas.

In our study, the high prevalence and wide geographical distribution of seroreactors in ELISA (22.8%) clearly conflicts with the limited number of WB positive samples (0.3%). The low BB prevalence is also not consistent with the results of previous ELISA-based serosurveys carried out in Central and Southern Italy (Gentile et al. 2012, Rinaldi et al. 2013). In an inter-laboratory comparative study, high sensitivity and specificity were registered for many commercial and in-house ELISA tests (Garcia-Lunar et al. 2013). However, other authors documented a high rate of false positives in ELISA, and recommended the complementary use of robust confirmation tests on occasion of BB serosurveys (Cortes et al. 2006, Alvarez-Garcia et al. 2013a, Basso et al. 2013b). Similarly as our study, 10% of investigated cattle in Switzerland tested positive with a commercial ELISA but only 0.3% was later confirmed WB positive (Basso et al. 2013b). In Australia, the same commercial ELISA yielded 18% seropositive cattle but was not confirmed in WB, and the authors concluded that *B. besnoiti* was absent in the country (Nasir et al. 2012b). False-positive results may be due to cross-reactions with closely related Apicomplexa. such as *Sarcocystis* spp., *Toxoplasma gondii* and *Neospora caninum* that are known to potentially cross-react with *Besnoitia* spp. (Shkap et al. 2002, Cortes et al. 2006, Fernandez-Garcia et al. 2009, Schares et al. 2010, Schares et al.

2011). Furthermore, in the present study most ELISA positive sera had RIPC values comprised between 9.7 and 20, suggesting low antibody titres in the majority of seroreactors. As opposite, most sera which were analyzed with the same ELISA in a BB endemic area in Spain showed RIPC values comprised between 20 and 80 (Gutierrez-Exposito et al. 2014).

Further, we analyzed a panel of sera by IFAT and a comparison with results obtained in WB was performed. IFAT showed a very low sensitivity (44.4%) and PP value (50%) demonstrating that in the surveyed area this serological test could be poorly able to detect all true positive sera. Otherwise, IFAT allowed increase specificity (92.3%) avoiding thus a major number of false positive in comparison to ELISA. Then, according to Basso et al (2013), both IFAT and the ELISA test could be used for screening purposes, with confirmation of positive results by WB. However, ELISA is more adequate for large screening whereas IFAT for analysis at individual level. IFAT was confirmed as a more time consuming and a more subjective technique in comparison to ELISA and the choice of coupling a standardized ELISA with a confirmatory WB resulted reasonable as a strategy to mostly carry out epidemiological studies at large scale in not endemic areas (Cortes et al. 2006, Alvarez-Garcia et al. 2013a).

In spite of the limited geographical distribution of *B. besnoiti* in the investigated areas, its circulation was intense in infected farms A, where repeated clinical cases have been recorded in a relatively short time interval (Gazzonis et al. 2014). In this farm, the 22% prevalence of ELISA seroreactors, later confirmed by WB, compares favorably with similar screenings carried out in outbreak farms in Italy (Gentile et al. 2012) and other not endemic areas in Europe (Alvarez-Garcia et al. 2013a, Hornok et al. 2014). In farms like this, testing for BB should be mandatory on new entries and on the whole herd in order to control the diffusion of infection in and outside the farm. According to Alvarez-Garcia et al. (Alvarez-Garcia et al. 2013a), a few measures as the employment of seronegative bulls in natural mating and culling of seropositive or with clinical signs animals should be adopted to an effective control of this infection.

Two out of five confirmed positive farms housed Holstein Fresians. Most BB outbreaks in Europe were recorded in beef farms and most serosurveys carried out so far focused on beef cattle (Schares et al. 2009, Gollnick et al. 2010, Manuali et al. 2011, Mutinelli et al. 2011, Gentile et al. 2012, Basso et al. 2013b, Hornok et al. 2014). However, a higher susceptibility of beef cattle to *Besnoitia* infection has not been demonstrated (Jacquiet et al. 2010, Rinaldi et al. 2013). It is reasonable to assume that beef cattle, which are more frequently raised outdoor, are at greater risk of exposure to the bite of putative insect vectors (Lienard et al. 2011).

Transmission through direct contact during natural mating has been also hypothesized (Alvarez-Garcia et al. 2013a, Esteban-Gil et al. 2014). In farm A, where natural mating is practiced, bulls in service were imported from BB endemic areas abroad.

Transportation of cattle between areas and countries is a well recognized risk factor for BB (Alvarez-Garcia et al. 2013a). In the present survey, only three positive animals were imported (Farm A) but the origin of the infection may not be inferred, since we could not know whether the animals were already immunized or if they acquired the infection once in Italy, although it is reasonable to infer that the *Besnoitia* infection could be related to the import of subclinically infected cattle into a farm followed by local transmission. Moreover, farm B was a lairage: in this kind of farms animals from different origins are joined and this increases the sanitary risks, making possible to be infected. Regarding the other positive animals born in Italy, it has to be considered the possibility of be infected through the contact with imported animals but also through other ways of transmission such as mechanical vectors. In fact, the location of the positive farms is quite near the Apennine Mountains, suggesting that the area could represent an ideal habitat for insect vectors contributing to spread the infection in Italy.

8. Case report on a naturally infected herd: serological and histopathological aspects in an infected herd

8.1. Introduction

Considered endemic in Spain, Portugal and France and emerging in Europe (EFSA 2010), bovine besnotiosis is already considered endemic in certain areas of Central Italy, where some outbreaks of infection have been diagnosed. Moreover, a serological study revealed high prevalence of infection (83% herd level; 44.1% individual level) in a southern region.

Our serological data in northern Italy and Sardinia island showed a lower seroprevalence values, (P=0.3%) with the recording of some foci of infection in Lombardy region.

Bovine besnoitiosis is responsible of economic losses due to mortality, decrease in milk production and transitory or permanent sterility in males (EFSA 2010). The disease in cattle may present subclinically or clinically, severe but usually not fatal. In the acute phase, animals may show fever, lymphadenitis, subcutaneous oedema, loss of body condition and testicular inflammation. Clinical signs may not be noticed or not associated to besnoitiosis, since not specific. Subsequently, during the chronic stage a gradual deterioration of body condition and loss of body weight occur; bradyzoites organize in cysts in subcutaneous tissue, skin, mucosal membranes and sclera conjunctiva. In this phase the pathognomonic subcutaneous thick-walled tissue cysts appear, with thickening and folding of the skin and hypotrichia or alopecia (Alvarez-Garcia et al. 2013a). Cysts were described in several target organs (particularly skin, mucosae, upper respiratory tract, and genital tract), although only few studies have been carried out on alteration caused by *B. besnoiti* from a histological point of view (Langenmayer et al. 2012, Lopez et al. 2012, Frey et al. 2013).

Therefore, the aim of the present survey was to describe an outbreak of bovine besnoitiosis in an infected beef farm in Lombardy. We investigated the serological state of the farm and described the clinical outcome of the disease in six clinical cases from a histopathological point of view.

8.2. Materials and methods

Herd description and blood sampling. The surveyed farm was located in the southern area of Lombardy region quite near to Apennine Mountains. It was a beef farm housing about 700 Limousine cows over 2 years and 28 breeding bulls. Cows were used for reproduction and calves were nursed only usually for 6-7 months after calving. Most bulls in service were imported from France (19 out of 28); the remaining nine bulls were born in farm and used as breeding studs. Both

artificial insemination and natural mating were practiced. Animals were housed in groups of 10 individuals with possibility to go outdoors; groups were not fixed and animals were moved according to management necessities. Moreover, one year before the survey the farmer bought 20 animals from a farm located in the same municipality that had previously imported a batch of cattle from France. In the month of October 2013, 544 animals over 2 years were randomly selected and sampled; all 28 males were included in the sampling. The age of sampled animals was less than 8 year in 157 animals, it was comprised between 8 and 12 in 185 ones, and 202 animals were older than 12 year old. Blood samples were collected from jugular or tail vein, conserved in tubes without anticoagulant agents and transported to laboratory in few hours, then centrifuged (15 min, 2120 g). Sera were stored at -20°C until analysis. At sampling time, individual data on each sampled animal were collected. Information on animal movement was provided by the owner and by the Italian National Data Bank for Zootechnical Registration (https://www.vetinfo.sanita.it/). Particularly, out of the 544 sampled ones, 348 cattle were imported from abroad (France, Netherlands and Luxembourg) whereas only 196 were born in farm or in another Italian farm.

Serological analysis. Samples were analyzed using a standardized in house-ELISA as described by (Fernandez-Garcia et al. 2010). Absorbance was measured as optical density (OD) values at 405nm using a microplate reader (Multiscan RC 6.0; Labsystems). Samples were analyzed in duplicate, and the mean value of the OD was converted into a relative index per cent (RIPC) by employing the following formula:

RIPC = (OD sample - OD-negative control)/(OD-positive control - OD405-negative control) × 100.

Samples with an RIPC ≥ 9.7 were considered positive.

Subsequently, to confirm positive samples, positive samples were re-tested by a tachyzoite-based Western Blot (WB) performed under non-reducing conditions according to (Fernandez-Garcia et al. 2009). Apparent prevalence (AP) and true prevalence (TP) were calculated for both tests (Greiner and Gardner 2000) basing on values of sensitivity and specificity reported by (Garcia-Lunar et al. 2013): 97.3% sensitivity (Se) and 94.6% specificity (Sp) for ELISA and 97.3% Se and 94.6% Sp for WB.

Statistical analysis. To evaluate the agreement between ELISA and WB results, Kappa statistic was calculated. Data obtained by WB were analyzed by a multivariate logistic regression analysis using as independent variables the following information: gender, age (computed in months) and origin (born in Italy or imported from abroad). The model was developed by backward elimination until all remaining variables were significant ($P<0.05$). Goodness of the model fitness was assessed by the Hosmer-Lemeshow statistic. Statistical analysis was performed with SPSS (version 19.0; SPSS, Chicago, IL).

Clinical examinations of symptomatic animals and tissue sampling. During a period comprised between May and October 2013, six animals (four cows, one heifer and a bull) presented symptoms ascribable to bovine besnoitiosis. Clinical examinations and animals monitoring were performed by the farm veterinarian.

Microscopic examination for tissue cysts. For the direct visualization of the parasitic cysts on the skin biopsies of two cows and the bull, small pieces of tissue were squashed between trichinelloscopy plates and analyzed by direct microscopic examination.

Histology and immunohistochemistry. Histological examination was conducted on skin punch biopsies of the cows and the heifer and on a variety of tissue samples of the bull, including skin of the thigh, neck, eyelid and muzzle, scleral conjunctiva, mucous membranes of the upper respiratory tract, pharynx, liver, cardiac muscle, lungs, testes, mediastinal lymph nodes. Tissue specimens were fixed in 10% buffered formalin, embedded in paraffin-wax, sectioned at 5 µm, stained with hematoxylin and eosin (HE) and examined by microscopically. Immunohistochemical staining was performed on serial sections from formalin-fixed paraffin-embedded tissue with the avidin-biotin-peroxidase technique using goat anti-rabbit biotinylated antibody (BA-1000) and avidin biotin complex reagents (Vectastain Elite ABC kit) obtained from Vector Laboratories, (Burlingame, CA). A polyclonal rabbit serum against *Besnoitia besnoiti* tachyzoites provided by SALUVET was used at 1:80,000 dilutions primary antibody after pepsin enzymatic pre-treatment.

8.3. Results

Serology. Among the 544 sera tested with the ELISA, 122 of these were resulted positive, with an AP of 22.4% (95% CI: 18.9-25.9); TP was calculated at 18.3% (95% CI=15.1-21.5). Considering data obtained with WB analysis, the test confirmed 100 out of the 122 tested (AP=18.3%; 95% CI= 15.1-21.6); TP was calculated at 16.7% (95% CI: 13.6-19.9). Considering values obtained in ELISA, RIPC results were resumed in Table1. As described in

, the major part of seroreactors in ELISA confirmed by WB presented RIPC values greater than 20, whereas only 25 samples out of 44 ones were confirmed positive by WB. To state the agreement between ELISA and WB results, a Kappa statistic was performed and a 0.865 value was obtained (excellent agreement). The percentage of false positive in ELISA but negative to WB was calculated at 18%. The percentage of false positive is higher in the samples with ELISA RIPC values comprised between the cut-off and 20 (43.1%), whereas with higher RIPC values the percentage decreases (Table 32).

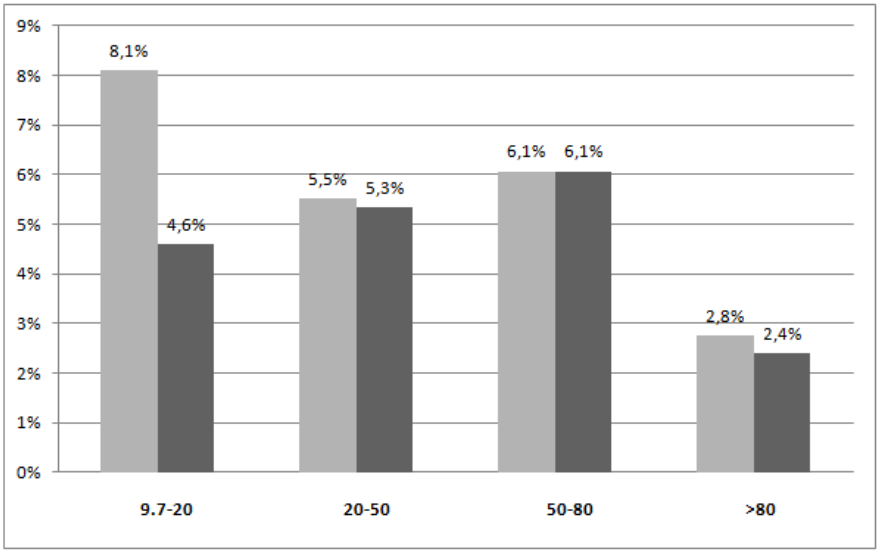

Fig. 21 - Antibody levels distribution (RIPC values) on seropositive animals in ELISA (grey) and subsequent WB (black). Percentage is calculated on the total number of sampled animals (n=544).

Table 32 - Antibody levels distribution (RIPC values) on seropositive animals in ELISA and subsequent WB. Percentage is calculated on the total number of sampled animals (n=544).

RIPC values	+ ELISA	% + ELISA	+ WB	% + WB	% of false positive
9.7-20	44	8.1%	25	4.6%	43.1
20-50	30	5.5%	29	5.3%	3.3
50-80	33	6.1%	33	6.1%	0
>80	15	2.8%	13	2.4%	13.3
Overall	122	22.4	100	18.3	18

Results obtained from risk factor multivariate analysis are resumed in Table 33. Fitness model was calculated at Goodness of fit was assessed by the Hosmer-Lemeshow=16.247 with a *p*-value=0.039.In particular, although considerably fewer in comparison to females in our survey, males have a risk of infection 3.3 times higher than females. Age represent an important risk factor, increasing the risk of infection with a factor of 1.015 for an increase of age of 1 month. The variable "origin" (born in Italy or imported from abroad) did not result statistically significant and was removed from the model. Nevertheless, a slightly higher number of cattle imported from abroad resulted seropositive (66 positive out of 348 samples; P=19.0%) than those born in Italy (34 positive out of 196; P=17.3%).

Table 33 - Potential risk factors associated with B. besnoiti seropositivity to ELISA test in cattle using multivariate model.

Variable	Category	n	Prevalence (%)	Odds ratio	95 % CI	p-value
Age	Continuous variable	544		1.015	1.007-1.023	<0.001

Sex	Females (reference)	517	17.8			
	Males	27	29.6	3.300	1.322-8.241	0.011

Statistically significant variables are indicated by bold typing

Clinical examinations of symptomatic animals. At the end of May 2013, four cows of the herd presented clinical symptoms of acute stage of besnoitiosis with hyperthermia, photophobia, epiphora, nasal discharge and dispnea; further, they showed oedematous swellings (anasarca) of head, limbs, and lower parts of the body. Two of these cows presented also scleropachidermia, alopecia and subcotaneus nodules (chronic stage) restricted to the posterior surface of the thigh. A 5 year-old bull was slaughtered in September with the clinical signs of the chronic form of the disease. The skin was at length altered and characterized by thickening, hardening, folding and wrinkling of the skin, with the lesions distributed at the head, neck, thorax, abdomen, legs and scrotum. The bull was depressed and with anamnesis of anorexia. At the end of October, a heifer presented pearly-white, elliptic, and relieved cysts on the mucous membrane of vaginal vestibule, without any alteration of the skin. All of these animals showed seroreaction both in ELISA and in WB, with RIPC ELISA values ranging from 45.2 to 191.8.

Table 34 - Seropositive animals submitted to clinical examination presenting symptoms of bovine besnotiosis and sampled for histology/himmunohystochemistry.

Animal ID	Age (years)	origin	Time in farm (years)	symptoms	RIPC ELISA	WB	Histology[a]
Cow 1	8	France	6	Acute symptoms, oedematous swellings	98.6	+	Cutaneous biopsy punch
Cow 2	8	France	6	Acute symptoms, oedematous swellings	80.6	+	Cutaneous biopsy punch
Cow 3	9	Italy	9	Acute symptoms, oedematous swellings, scleropachidermia and subcotaneus nodules	45.2	+	Cutaneous biopsy punch +
Cow 4	9	Italy	9	Acute symptoms, oedematous swellings, scleropachidermia and subcotaneus nodules	62.2	+	Cutaneous biopsy punch +
Bull	5	Italy	5	Chronic symptomps, depression, anorexia	191.8	+	Cutaneous biopsy punch +, skin of the thigh+, neck+, eyelid+ and muzzle+, scleral conjunctiva+, mucous membranes of the upper respiratory tract+, pharynx+, liver, cardiac muscle, lungs, testes+, mediastinal lymph nodes
Heifer	1.5	France	0.5	Cysts on vaginal mucosae	117.4	+	Cutaneous biopsy punch

[a] specimens presenting cysts are indicated with "+"

Microscopic examination for tissue cysts. From skin biopsies analyzed by direct microscopic examination with trichinelloscopy plates, tissue cysts containing bradizoytes attributable to *B. besnoiti* were found in the cutaneous samples of the bull and cow 3 and 4 (Fig. 22).

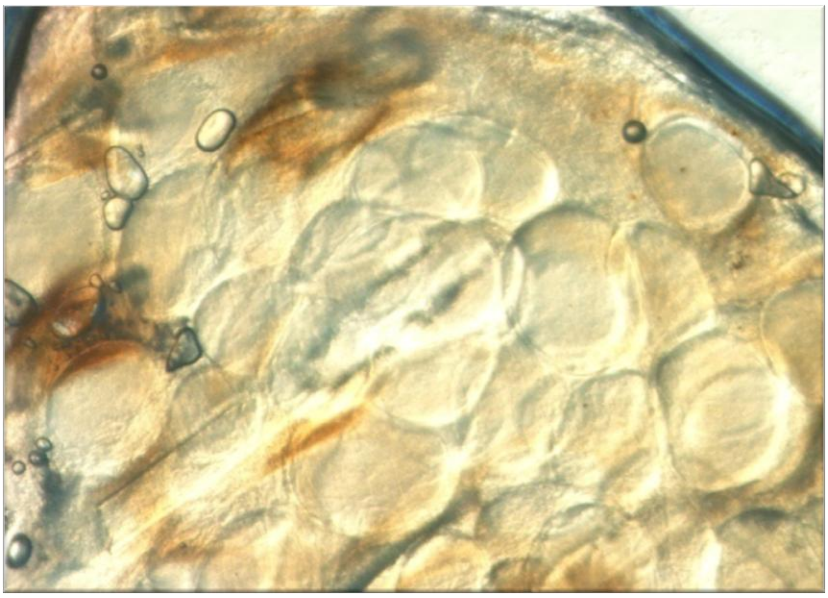

Fig. 22 - Direct microscopic examination of tissue cysts through their squashing between trichinelloscopy plates.

Istology and immunohistochemistry. In skin punch biopsies, numerous large, round to oval well developed cysts morphologically consistent with *Besnoitia* spp. were present in the two cows. Cysts were not detected in skin punch biopsies of the heifer. Parasitic cysts had a maximum diameter of about 400 µm and 5 to 20 µm thick wall and contained numerous densely-packed spindle-shaped bradizoites. Infected host cells, the majority fibroblasts or myofibroblasts, were surrounded by a layer of hyaline connective tissue and had one or more hypertrophic nuclei peripherically displaced by the parasitophorus vacuole. In tissue samples of the bull, the highest concentration of Besnoitia cyst was seen in the skin and mucosal membranes of pharynx, larynx and trachea and fewer cysts were present in eyelid dermis, scleral conjunctiva, testicular parenchyma and in the muscular wall of blood vessels. No *Besnoitia* cysts were detected in lungs, liver, heart and mediastinal lymph nodes. A few early developing *Besnoitia* cysts were also found in positive samples. They were smaller (approximately 60 to 100 µm) and appeared as large hypertrophic host cells with one or more large nuclei and a parasitophorus vacuole containing a variable number of bradizoites. With regard to tissue distribution, in skin and mucosae, *Besnoitia* cysts were mainly located in superficial dermis and mucosal lamina propria, often grouped in clusters. Cysts were frequently surrounded by a mixed non-suppurative inflammatory infiltration of lymphocytes, macrophages and rare eosinophils. Occasionally, microgranulomatous reaction was also found. In the skin, hyperkeratosis

and moderate acanthosis and superficial dermal fibrosis were also present. Testicular atrophy, fibrosclerosis and moderate multifocal perivascular and interstitial chronic lymphohistiocytic infiltration accompanied Besnoitia infection in the testis.

By immunohistochemistry, both bradyzoites in the parasitophorus vacuole and the host-cell cytoplasm reacted positively to rabbit serum against *Besnoitia* tachyzoites. Positive cysts were seen only in HE positive samples.

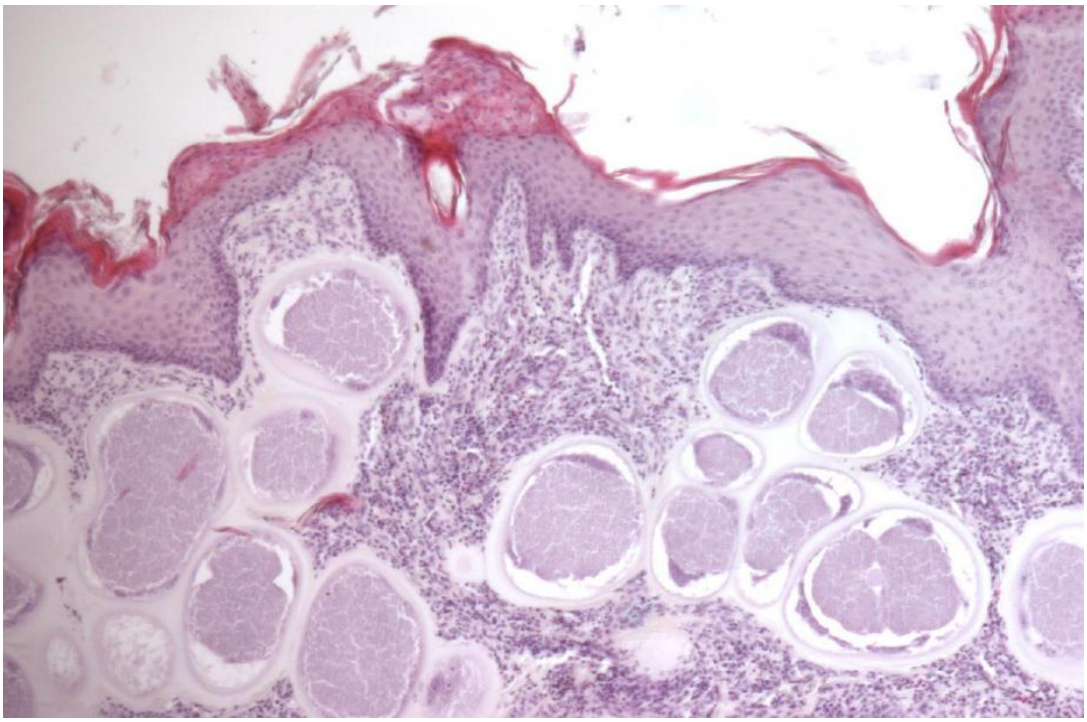

Fig. 23 – Histological section of skin: hyperkeratosis with lymphohistiocytic and eosinophilic flogosis.

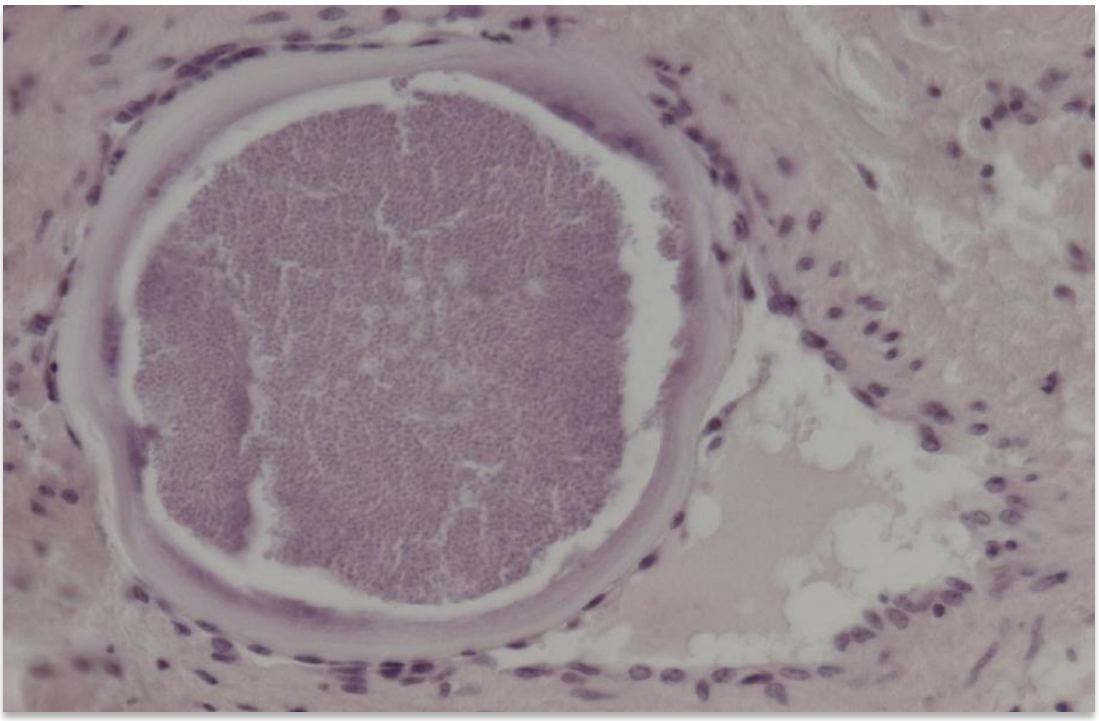

Fig. 24 – Histological section of a blood vessel: parasitic cyst developed in the lumen starting from tunica media.

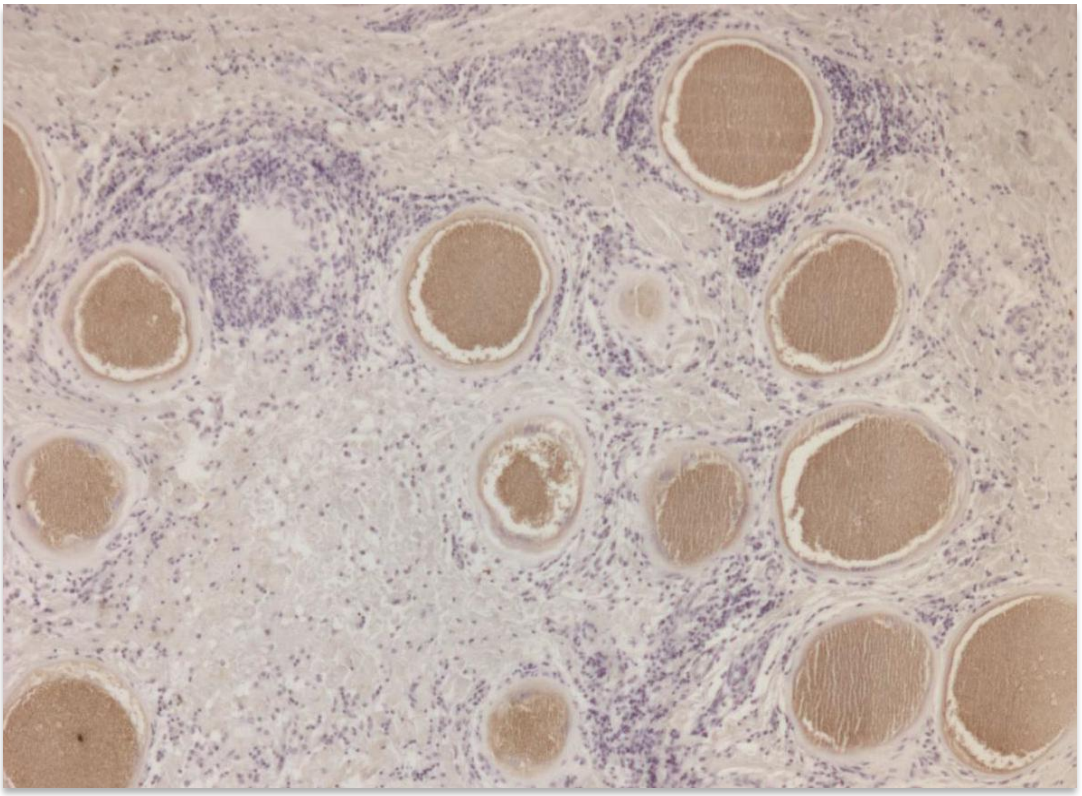

Fig. 25 – Section of pharyngeal mucosa in immunohistochemistry.

8.4. Discussion

In the present study, we reported a picture of *Besnoitia besnoiti* infection in a beef farm from a clinical, serological and histopathological point of view. The investigated farm was located in Northern Italy (Lombardy region) quite near (232 km) to the Apennine Mountains where an autochthonous outbreak of bovine besnoitiosis was recently detected in a dairy farm (Gentile et al. 2012). This report confirms the circulation of *B. besnoiti* in beef and dairy cattle in Italy and this is the first record of bovine besnoitiosis in autochthonous beef cattle. *B. besnoiti* infection was confirmed in 100 out of 544 cattle from the tested herd (AP=18.3%; TP=16.7%); (Jacquiet et al. 2010) suggested that in an area in which bovine besnoitiosis had not been previously reported, the number of seropositive animals may be quite high after the detection of the first cases. Further, our prevalence value is similar to the lowest intra-herd prevalence (15.1%) found by (Gutierrez-Exposito et al. 2014) in a large-scale survey regarding beef cattle from the Spanish Pyrenees, an area endemic for bovine besnoitiosis. Vice-versa, our result is quite higher than the intra-herd prevalence value (9.7%) previously recorded in Holstein cattle from an infected farm in the Italian Apennines (Gentile et al. 2012).

Cattle from beef farms seem to be at a higher risk of infection in comparison to dairy cattle, as demonstrated by several epidemiological surveys (EFSA 2010, Alvarez-Garcia et al. 2013a). Further, the major part of foci of infection in Europe have been registered in beef farms and, as a consequence, serological survey focused so far on beef cattle (Schares et al. 2009, Gollnick et al. 2010, Manuali et al. 2011, Mutinelli et al. 2011, Gentile et al. 2012, Basso et al. 2013b, Hornok et al. 2014). Although a greater susceptibility to besnoitiosis in beef cattle has not been proved (Jacquiet et al. 2010, Rinaldi et al. 2013), differences in management practice may lead to a major risk of acquire the infection: as an example, beef cattle, frequently raised outdoor, may be more exposed to the bite of insect vectors (Lienard et al. 2011). Further, sexual transmission via natural mating, more frequently practiced in beef farms than dairy ones, has been also hypothesized (Alvarez-Garcia et al. 2013a, Esteban-Gil et al. 2014).

As suggested by several authors, one of the major risk factor is the animal trade between countries (Alvarez-Garcia et al. 2013a); previous studies reported the infection in imported cattle (Basso et al. 2013b, Hornok et al. 2014). Nevertheless, in our survey, even if in the surveyed herd a major number of cattle (63.9%) were purchased abroad, no difference was registered in seroprevalence between autochthonous or imported animals ($p>0.05$). Indeed in the studied farm among the selected cattle 348 were imported from abroad (mainly France, but also Luxemburg and Netherland) and only 196 were born in farm or in another Italian farm. Furthermore, the farmer reported the buying in 2012 of a stock of 20 cows from a neighbouring beef farm that was going to

move to France. Subsequently, once in France, the French National Veterinary Service tested the introduced cows by serology resulting seropositive to BB (personal communication). However, since previous clinical or serological data are lacking, it is not possible to interfere whether BB was introduced in farm with the buying of the group of cattle, although is a reasonable hypothesis.

Moreover, it should be also considered that the surveyed herd was composed by cattle proceeding from several European countries, France included, and bulls in service are regularly introduced from abroad. Further, the rate of animal handling in the farm is very important; bulls or cows were sold in or outside Italy and a few animals were regularly moved to exhibit them in cattle fair in Italy or abroad. Therefore good management practices, such as serological testing of introduced animals, are essential to limit introduction and spread of BB in beef herds.

The present study showed that gender was the strongest predictor of infection for the cattle. Bulls were more at risk of be found seropositive (OR=3.300) than females. Several studies demonstrated that males showed a higher sensibility to the infection both in terms of serological evidences and clinical signs, which are described more severe in bulls than in cows (Jacquiet et al. 2010, Alvarez-Garcia et al. 2013a, Gutierrez-Exposito et al. 2014). This was also proved by the outcome of the clinical cases followed in the present survey: in comparison to the cows with clinical lesions, the slaughtered bull presented more severe symptoms, with cutaneous lesions all over the body and with more severe systematic damages. Besides, the loss of the bull had a greater the economical impact for the farmer, since it could not be used for reproduction due to the infertility caused by besnotiosis.

Animal age has been reported to be a risk factor by other serological surveys showing that the seroprevalence increase with age (Fernandez-Garcia et al. 2010). We reported an increasing of the risk of infection (OR=1.015) with the increasing of the age. The appearance of acute clinical signs and the presence of chronic lesions in infected cattle are more frequent in adult animals. Older animals could become easily infected due to longer periods of exposure than the younger, explaining therefore the phenomenon. Also considering clinical cases observed in the farm, animal appear to develop symptoms in adulthood, as reported to other authors (Alvarez-Garcia et al. 2013a): the seropositive animals, which lesions were confirmed by histology and immunohistochemistry, had an age comprised between 5 and 9 years, with the exception of the heifer (18 months).

Regarding the methodological aspects, serological examination was carried out using two standardized test: an ELISA for the first screening, and a WB for the confirmation of the positive samples. With the combination of at least two subsequent serological test specificity increases, and it allow to obtain more robust results, especially in not-endemic areas, as suggested by several

authors (Cortes et al. 2006, Alvarez-Garcia et al. 2013a, Basso et al. 2013b). Nevertheless, in house-ELISA revealed an efficient test for a mass-screening, as confirmed by the good values of sensitivity and specificity (Garcia-Lunar et al. 2013) and by the good agreement with results obtained by WB (K test=0.865).

Considering anatomopathological data, the use of trichinelloscopy for the miscroscopic detection of tissue cysts revealed a tool for a fast and economically faceable diagnosis, even if the diagnosis of BB has to be confirmed with other methods (i.e. histology or molecular biology). Histology and immunohystochemistry revealed useful tool in the diagnosis of BB in seropositive animals presenting clinical signs of scleropachidermia. Among the six animals investigated, all of them resulted positive in serology whereas only the bull and two cows showed cysts containing *B. besnoiti* bradizoytes. The other two cows, showing the acute symptoms, and the heifer, having only nodules in the vulvar region, did not present any cyst ascribable to *B. besnoiti*; possibly, they could be sampled still in the early phases of the infection. It would be interesting to keep monitoring these animals in order to detect possible chronic signs of BB.

Skin and mucosae of upper respiratory tract contained the highest number of *B. besnoiti* cysts, as well as endothelium, as previously recoreded by several authors (Jacquiet et al. 2010, Rostaher et al. 2010, Manuali et al. 2011). Cysts were also recorded in rhynarium mucosae. Differently from other recordings, no cysts were recorded in certain target organs such as liver and hearth (Jacquiet et al. 2010). Considering testis, the finding of cysts in the parenchyma is in agreement with several surveys (Alvarez-Garcia et al. 2013a); testis showed an atrophic parenchyma caused by the compression by cysts on tubules and seminiferous epithelium.

Immunohystochemistry confirmed *B. besnoiti* bradizoytes in cysts in all specimens, confirming histological diagnosis. (Frey et al. 2013) showed on the contrary as immunohistochemistry may highlight lesions not visible with EE coloration, increasing thus the sensitivity of the techniques.

General conclusions

Among Apicomplexa, particularly three protozoa (i.e. *Toxoplasma gondii*, *Neospora caninum* and *Besnoitia besnoiti*) were selected in the current studies. All of them are nowadays considered (re-) emerging and under-reported in Europe but they are proven to have important consequences on public health and food security, in addition to animal welfare and health. Moreover, an economical aspect concerning zootechnical activities is to be highlighted: *T. gondii* and *N. caninum* are considered major infective cause of reproductive failure in small ruminants, with huge economic impact, although few data are available mostly on neosporosis in small ruminants (Ortega-Mora et al. 2007). Similarly, bovine besnoitiosis is responsible of economic losses in affected herds (Cortes et al. 2005, Jacquiet et al. 2010) and, according to (EFSA 2010), epidemiological surveys are necessary to monitor the presence of *B. besnoiti* in Europe.
Therefore, the aim of my doctoral project was to investigate on the selected protozoan infections in small ruminants and cattle, following different research lines.

Over last years, the growing interest of consumers in food safety addressed research on focusing on pathogens threatens food safety. Among protozoan parasitic diseases potentially causing important food-borne disease, toxoplasmosis indeed represents a public health concern worldwide, although the disease has been neglected for a long time (Kijlstra and Jongert 2008). Although the consumption of uncooked or undercooked meat is considered one of the major risk factor for human infection, any specific control is mandatory for toxoplasmosis within the food chain, in spite of EFSA classification of *T. gondii* as a high priority for meat inspection mostly certain species (EFSA 2013). Moreover, from a zootechnical point of view, *Toxoplasma gondii* and *Neospora caninum* have been recognized as ones of the most important abortigenic pathogens both domestic ruminants. Updated epidemiological data on *T. gondii* infections in humans and animals are scarce and those existent lack in standardization and harmonization through countries.
Therefore, a serosurvey was carried out to update epidemiological data on *T. gondii* and *N. caninum* infections in small ruminants bred in Northern Italy (Research line 1). High seroprevalence values were registered for both protozoa at individual and farm level, consistent to those registered in previous survey carried out in European countries.
Managerial variables resulted associated to both infections, with traditional kind of farming more at risk of infection in comparison to intensive one, usually at a higher standard level of technology and hygiene. Further, spatial analysis was performed in order to determine geographical and climatic

features able to facilitate the maintenance of *T. gondii* and *N. caninum* infections within the study area. As an example, for *T. gondii* infection in goats an area of high risk of infection was identified and climatic variables, such as mild temperature and rainfall, resulted predictors of infection. Geospatial analysis and analysis on climatic data revealed useful tools for the identification of clusters of infection, although data obtained should be always interpreted considering individuals and herds factors.

Considering the whole serological results, a detailed description of both infections was depicted leading to an identification of variables involved in their maintenance and spread. Particularly for goats, small family-run farms resulted highly infected with *T. gondii* and *N. caninum*; this kind of zootechnical activity represents a traditional economical resource in certain mountain areas of Northern Italy, with the production of cheese and foodstuff not easily available on great food supply chains. Sanitary authorities should encourage and promote controls and monitoring of such pathogens, with the double aim of reducing infections within herds and safeguard public health.

Considering the high seroprevalence values registered in the epidemiological survey, a deeper investigation on *T. gondii* infection in small ruminants' foodstuff was planned. In research line 2, following EFSA guidelines (2010), we selected milk and meat as products potentially posing a risk for consumers.

Firstly, a commercial ELISA was validated to analyze goats' milk sample and it revealed as a useful tool for a first approach to the diagnosis of *T. gondii* infection at individual and farm level: collecting milk is easier and less expensive than collecting sera samples, as well as less stressing for the animals.

In a "case study" caprine farm naturally infected by *T. gondii*, variation in antibodies anti-*T. gondii* both in serum and milk during lactation was analyzed. Different curves of antibodies level in sera and milk were observed; from a diagnostic point of view, high IgG level in milk during the first fortnight from the parturition suggests this period as the fitter to sample and analyze milk.

Finally, two epidemiological surveys were carried out on ovine and caprine products: antibodies anti-*T. gondii* were detected in caprine bulk tank milk and in meat juice obtained by slaughtered goats and sheep, registering quite high prevalence values. Analysis on tank bulk milk revealed a fast and economically affordable tool in the context of a health monitoring program; it is to be intended as a first screening to point out farms having seropositive to *T. gondii* in dairy goats. In analogy, meat juice samples are easy to collect at slaughter and have been proven as suitable for the screening of infections in different meat-producing animals destined to human consumption, including *T. gondii*.

The proportion of seropositive animals in both surveys gives an idea of the circulating infection among small ruminants' populations and an estimation of the risk of infection for consumers, although the finding of seropositivity in milk and meat juice samples did not surely represent a hazard for food safety.

Finally, in research line 3, an epidemiological survey on bovine besnoitiosis was performed on dairy and beef cattle bred in northern Italy (Lombardy and Piedmont), Liguria region and Sardinia Island. Only five independent foci of infection were registered in Lombardy region. However, when a subsequent in-depth analysis was performed in a seropositive farm applying different diagnostic tools (serology, histology and immunohistochemistry), high intra-herd prevalence and several clinical cases were recorded. Bovine besnoitiosis was confirmed as a threatening disease and surveillance and monitoring should be planned particularly in not-endemic countries as Italy.

Concluding, my doctoral project allowed to update information on important protozoa considered (re-)emerging and under-reported. High values of *Toxoplasma gondii* and *Neospora caninum* seroprevalence were registered in small ruminants in Northern Italy; geospatial analysis revealed a useful tool for an inclusive comprehension of predictors involved in the infections.
T. gondii infection deserves a major attention considering the sanitary risk posed by caprine and ovine foodstuff. Alternatively to classic serological methods, analysis on individual and tank bulk milk and on meat juice samples were confirmed a valid tool for a fast and affordable screening of *T. gondii* infection in small ruminants. Finally, an important contribution to the epidemiology of an emerging disease as bovine besnoitiosis was given, with the update of epidemiological data and the deeper investigation on an infected farm, confirming the necessity to plan monitoring controls.
Further perspectives involve the molecular detection of *T. gondii* in milk and meat product and the genotyping of strains would allow clarifying the effective risk for food safety; in small ruminants indeed type II, considered the predominant lineage causing human toxoplasmosis, has been previously reported. In analogy, molecular analysis on different *B. besnoiti* isolated and on hematophagous insects, potential mechanical vectors of the infection, represents a challenge for a deeper comprehension of mechanism involved in the spread and transmission of the disease.

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